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SYMPOSIUM PAPER

The "A, B and C" of Her-2 DNA vaccine development

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Abstract
Introduction The development of Her-2 DNA vaccine has progressed through three phases that can be categorized as phase "A": the pursuit of Her-2 as a tumor-associated "antigen", phase "B": tilting the "balance" between tumor immunity and autoimmunity and phase "C": the on-going "clinical trials".
Materials and methods In phase "A", a panel of human ErbB-2 or Her-2 plasmids were constructed to encode non-transforming Her-2 derivatives. The immunogenicity and anti-tumor activity of Her-2 DNA vaccines were tested in human Her-2 transgenic mice with or without the depletion of regulatory T cells (Tregs). However, Treg depletion or other immune modulating regimens may increase the risk of autoimmunity. In phase "B", the balance between tumor immunity and autoimmunity was assessed by monitoring the development of experimental autoimmune thyroiditis (EAT). To test the efficacy of Her-2 DNA vaccines in cancer patients, clinical trials have been initiated in phase "C".
Results and conclusions Significant anti-Her-2 and anti-tumor activity was observed when Her-2 transgenic mice were electro-vaccinated after Treg depletion. Susceptibility to EAT was also enhanced by Treg depletion and there was mutual amplification between Her-2 immunity and EAT development. Although Tregs regulate both EAT and Her-2 immunity, their effector mechanisms may differ. It may be possible to amplify tumor immunity with improved strategies that can by-pass undue autoimmunity. Critical information will be revealed in the next decade to expedite the development of cancer vaccines.

Keywords Her-2 · ErbB-2 · DNA vaccine · Cancer vaccine · Autoimmunity

Introduction

The development of a cancer vaccine generally progresses through three phases that can be categorized as phases "A, B and C". In phase "A", the effort is focused on the "antigen" of interest. Much debate goes into choosing the target molecule, whereas the vaccine formulation may be based on rational design or technical feasibility. An appropriate animal model is a must and determines the validity of the experimental approach. A less considered, but equally important issue is the "balance" between the induction of tumor immunity and autoimmune diseases or phase "B" of the study. Since tolerance to tumor associated antigen (TAA) is profound, strenuous immune modulation is required to achieve meaningful response. A predictable side effect of such immune modulation is inadvertent reactivity to self-antigens. Yet, the balance between tumor immunity and autoimmunity is often considered in a cursory manner in pre-clinical studies and autoimmune side effects are treated symptomatically in patients undergoing immunotherapy. When a "clinical trial" of the candidate vaccine is initiated in phase "C", it can mark the beginning or the end of the effort. In this presentation, the "A, B and C" of Her-2

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DNA vaccine development is summarized with our current perspective.

Phase A: Her-2 as the vaccine antigen

In the late 1990s, we chose Her-2 as our vaccine target because of the amplified expression on tumor cells and oncogenic activity [1–5]. This choice is now supported by the efficacy of anti-Her-2 mAb Trastuzumab (Herceptin) in Her-2 positive breast cancer therapy [6]. Naked DNA was selected as the vaccine formulation because of its stable and well-defined nature and because humoral and cellular immune responses to the entire repertoire of antigenic epitopes could be induced. Since it is free of confounding foreign entities, DNA vaccines can be administered repeatedly without losing activity. The feasibility of producing large quantities of pure DNA with simple tools is another major advantage. From intramuscular injection of Her-2 DNA, the vaccine regimen has evolved to include both Her-2 and cytokine DNA administered together by electroporation after regulatory T cell depletion [5, 7–16]. The activity of this improved regimen exceeded our initial expectation and Her-2 DNA vaccine is showing promise as a clinical vaccine candidate.

Her-2 DNA vaccine constructs

A panel of human ErbB-2 (E2) DNA constructs have been generated to encode recombinant Her-2 proteins that are free of tyrosine kinase activity and traffic to specific subcellular compartments [5, 7, 17]. Of the four basic constructs, pE2A encodes the full length Her-2 with a single substitution of aa Lys753 to Ala753 to remove the ATP binding lysine residue. pE2TM encodes the signal peptide, extracellular and transmembrane domains without the intracellular domain (ICD). psecE2 encodes the N-terminus aa 1–505 of the extracellular domain as a secreted protein. These three constructs induced both cellular and humoral immune responses and strong protective immunity in BALB/c and C57BL/6 mice. Another construct pcytE2, with truncated ER signal peptide, directs the synthesis of a full-length, short-lived cytoplasmic protein, which is promptly degraded by the proteasome [7]. Accordingly, immunization with pcytE2 activates CD8 T cells without humoral response. In the early studies, Her-2 DNA alone was administered intramuscularly (i.m.) [5, 7, 17]. Later, pGM-CSF was added to the formulation and electroporation was administered at the vaccination site to enhance gene expression and immune reactivity [15, 16, 18].

Although significant Her-2 immunity with complete tumor rejection was achieved in wild type mice, it was necessary to further evaluate vaccine activity in animals that express human Her-2 as a self-antigen to mimic immune tolerance in humans.

Her-2 transgenic mice

Several transgenic mouse strains that express either wild type or mutant rat neu had been developed [19, 20]. Before human Her-2 transgenic mice were available, rat neu transgenic mice were the next best choice and tumors derived from these mice continue to serve important functions in biological studies. Assessment of reactivity against neu induced tumors in neu transgenic mice induced by heterologous human Her-2 vaccine [16], however, may not mimic the events in humans and the results may even be misleading.

To test Her-2 vaccines in a more relevant system, Her-2 transgenic mice were generated in our lab using whey acid protein (WAP) promoter to drive the wild type human ErbB-2 gene [11]. The mice have been back-crossed into C57BL/6 background and are maintained as herterozygotes because homozygous pups are never born. Spontaneous tumors have not been detected in any organs. C57BL/6 Her-2 transgenic mice produce little to no antibodies (Ab) or T cells when immunized with Her-2 DNA, showing their profound immune tolerance to Her-2 [11]. This poor responsiveness verifies Her-2 as a self-antigen and these mice are invaluable for testing candidate Her-2 vaccines. Because these mice do not develop spontaneous tumors, syngeneic tumors expressing human Her-2 by transfection are tested in Her-2 transgenic mice. Additional backcrosses into other mouse strains are underway and their immune responses to Her-2 DNA vaccine are being investigated.

On the occasions when Her-2/neu induced spontaneous mammary tumors are required, we use tumors from BALB NeuT mice, which express a transforming rat neu and develop spontaneous mammary tumors between 17 and 19 weeks of age. When it is necessary to test DNA vaccines in these mice, rat neu DNA is used. Several mammary tumor lines have been derived from NeuT spontaneous tumors, such as TUBO [20] and Bam1a [21] which grow progressively in NeuT as well as normal BALB/c mice. These cell lines are convenient test systems for Her-2/neu targeted therapies.

Amplification of immune reactivity to Her-2 by depleting regulatory T cells (Tregs)

The weak response of Her-2 transgenic mice to DNA vaccine may result from a reduced T cell receptor repertoire via negative selection in the thymus or suppression of immune reactivity by peripheral regulatory cells. With reports documenting suppressive activity of $CD4^+CD25^+$ Tregs in autoimmune diseases [22], we tested the effect of Treg depletion on tumor immunity. In the initial experiment, BALB/c mice were injected i.p. with CD25 mAb, PC61, either 5 and

6 days before or 1 and 3 days after s.c. inoculation with TUBO or D2F2/E2 cells. D2F2/E2 cells were hormone-induced BALB/c mammary tumor cells stably transfected with human Her-2. Tumors developed in all mice, but >90% of the tumors in Treg depleted mice regressed completely, even after they reached the size of 180 mm^3 [13, 15]. This dramatic tumor regression was induced simply by depleting Tregs, demonstrating in situ priming by a growing tumor. This finding challenges the long-standing paradigm that solid tumors are poor at priming the immune system, thus cannot be rejected effectively by immune cells. Rather, the immunogenicity of a tumor can be functional and powerful when the mice are free of negative regulation.

To test if anti-Her-2 immunity in tolerant mice was also under the control of Tregs [13–15], Her-2 transgenic mice were treated with anti-CD25 mAb 1 week before DNA electro-vaccination, then boosted three times every 2 weeks with the same DNA. Reduction in $CD4^+CD25hi$ cells in the peripheral blood was verified by flow cytometry. The levels of Her-2 Abs were enhanced from undetectable in mice with intact Tregs to 28 ± 27 μg/ml in Treg depleted mice. Therefore, Tregs suppressed Her-2/neu reactivity whether Her-2 was a foreign or self-antigen.

Antibodies (Abs) induced by Her-2 or neu DNA recognized only cognate antigen

After Her-2 and neu transgenic mice were both established, the activity of autologous versus heterologous Her-2/neu vaccines were tested. Wild type, Her-2 or neu transgenic mice were immunized with either human Her-2 or rat neu DNA vaccines. In normal BALB/c and C57BL/6 mice, immunization with Her-2 or neu DNA induced antigen specific IgG which did not cross-react with non-cognate Her-2 or neu [16]. This non-cross-reactive humoral response held true in Her-2 and neu transgenic mice [16]. Vaccination of Her-2 transgenic mice with self Her-2, but not neu induced anti-Her-2 Abs. Similarly, vaccination of NeuT transgenic mice with self neu but not heterologous Her-2 DNA induced anti-neu Abs.

T cells activated by Her-2 or neu DNA exhibited limited cross-reactivity

Compared to humoral response, T cell response induced by heterologous Her-2/neu was more promiscuous. After neu DNA vaccination, BALB/c mice generated T cells that recognized both Her-2 and neu and they rejected D2F2 tumors expressing either Her-2 or neu, showing T cell cross-reactivity when Her-2 and neu were foreign antigens. When NeuT mice were immunized with heterologous pE2TM, splenocytes reacted strongly to human Her-2 and moderately to self rat neu, with anti-neu reactivity at comparable or lower level than that induced by autologous neu DNA vaccine. Conversely, when Her-2 transgenic mice were immunized with heterologous pneuTM, neu-reactive T cells were generated, but they did not recognize autologous Her-2. Therefore, heterologous Her-2/neu vaccines induced Ab to cognate antigen only and induced T cells with varying levels of cross-reactivity to autologous antigens.

In NeuT mice, autologous pneuTM immunization induced T cells that reacted with neu expressing cells, but not the dominant neu epitope, N63, indicating a reduced or altered T cell repertoire in the tolerant host. This finding is consistent with the reported recognition of sub-dominant epitopes [23] or the presence of low avidity CD8 T cells [24] in the immunized FVBneuN transgenic mice. Similarly, CEA transgenic mice also recognized sub-dominant CEA epitopes and this was associated with the expression of CEA in thymic epithelial cells [25].

There have been a number of unidirectional studies on human Her-2 vaccines in rat neu transgenic mice. Immunization of NeuT mice with an adenoviral vector encoding Her-2 induced low-level cross-reactive Abs to neu and delayed tumor formation [26]. The adenoviral vector may induce a broader spectrum of Abs than naked DNA, thus the modest cross-reactivity with neu. In FVBneuN mice, spontaneous tumorigenesis was reduced by immunization with Her-2 DNA every month for 10 months [27]. It is possible that 10 monthly vaccinations with heterologous Her-2 induced enough cross-reactive T cells to autologous neu to exert anti-tumor effect. Tegerstedt et al. [28] reported delay of spontaneous tumorigenesis in BALB NeuT mice by immunization with a single dose of virus-like particle (VLP) displaying heterologous Her-2. It was unclear if cross-reactive Ab or T cells mediated the activity. In most reported vaccine studies including our own, spontaneous tumorigenesis in NeuT mice was controlled primarily by Ab, because NeuT tumors were highly sensitive to Ab even when T cell activity was too low to make an impact [16]. The sensitivity of NeuT tumors to anti-neu antibodies may reflect their exquisite dependence on neu-mediated signals for survival and proliferation.

Her-2/neu model systems recommended for vaccine studies

The limited and variable cross-reactivity between Her-2 and neu responses highlights the importance of using human Her-2 transgenic mice to evaluate human Her-2 vaccines in pre-clinical studies. Rat neu transgenic mice are valuable for establishing the principles of Her-2/neu targeted therapy, but should be used with caution in human vaccine development. Furthermore, all Her-2/neu positive tumor cells are not equally sensitive to Ab treatment. With the increasing use of Her-2 targeted drugs such as Herceptin or tyrosine kinase inhibitors, the surviving tumor cells

may become more resilient, requiring stronger immune modulation to exert anti-tumor immunity.

Phase B: "balance" tumor immunity with autoimmunity

Autoimmune thyroiditis

To address autoimmune side-effects during cancer immunotherapy, the induction of experimental autoimmune thyroiditis (EAT), a model of Hashimoto's thyroiditis, was chosen as the indicator of inadvertent autoimmunity. Hashimoto's thyroiditis, a hypothyroid syndrome, is one of the most prevalent autoimmune manifestations in humans, with 45% of women and 20% of men in USA showing focal thyroiditis at routine autopsy, although only 1% of women and 0.5% of men exhibit clinical symptoms. It is characterized by mononuclear cell infiltration and destruction of the thyroid, elevation of thyroid-stimulating hormone and decrease of thyroid hormones (T3 and T4). The production of autoantibodies to thyroglobulin and thyroid peroxidase [29], and T cell activation by thyroid antigens [30] are indicators of autoreactivity. Susceptibility to thyroiditis is strongly influenced by the haplotype of class II MHC [31]. For example, human HLA-DRB1*0301 (DR3) or murine H2k confers susceptibility to autoimmune thyroiditis [32, 33], whereas C57BL/6 H2b and BALB/c H2d are associated with resistance [34]. DR3, expressed in 24% of normal individuals, is also associated with type 1 diabetes [35] with approximately 20% of type 1 diabetic patients also developing autoimmune thyroid disease [36]. In susceptible individuals, clinical symptoms of thyroiditis are precipitated by endogenous or environmental factors, such as hormone stimulation, dietary iodide level, infection, irradiation, immunostimulatory cytokines, etc., some of these factors are consequences of cancer immunotherapy.

EAT is a well-studied mouse model and is inducible by immunization with mouse thyroglobulin (mTg), the 660 kD storage protein for iodinated thyroid hormone. To simulate humans with circulating thyroid antigens and experiencing various triggering factors, mouse EAT is induced by injecting mTg with an adjuvant such as LPS, or by repeated injections of soluble mTg [15, 37, 38]. Although thyroiditis patients can be treated effectively by hormone replacement therapy, EAT serves as a well-defined experimental system for mechanistic analysis and is a sensitive read-out of autoimmune complications in cancer therapy [39–41].

Tregs in EAT

Removal of suppressor T cells by early thymectomy and/or irradiation leading to low incidence of autoimmune diseases, including thyroiditis and gastritis, was shown in the mid-70s and $CD4^+$ T cells were shown to mediate tolerance in EAT [42]. The CD25 marker enables the separation of Tregs [43, 44]. Removal of $CD25^+$ Tregs enabled EAT resistant mice to develop thyroiditis upon stimulation and abrogated induced tolerance to EAT [15, 44, 45]. Other immune modulating agents, such as antagonist anti-CTLA-4 (Kong, unpublished), agonist anti-CD137 [44] or anti-GITR mAb [46] interfered with the establishment of EAT tolerance. During long-term cancer immunotherapy with repeated blockade of Tregs and/or co-stimulation of T effctors, different autoimmune diseases could manifest themselves [47–52].

Concurrent induction of tumor immunity and autoimmunity by Treg depletion

In BALB/c mice inoculated with TUBO tumor, treatment with anti-CD25 mAb induced tumor regression and neu specific T cells [15]. When the same mice received mTg by chronic i.v. injections, they produced mTg specific Ab and T cells with inflammatory infiltration in the thyroids [15]. The immune responses to neu or mTg were greater in mice undergoing TUBO tumor rejection and mTg injection than in those experiencing either alone. This was the first experimental study to assess amplification of tumor immunity and possible exacerbation of auto-reactivity. To further analyze the concurrent immune responses to Her-2/neu and mTg, we examined genetic regulation of the response in Her-2 tolerant mice.

Genetic regulation of tumor immunity and autoimmunity

A mouse model that expressed both mTg and human Her-2 as self-antigens and expressed HLA-DR3 was established to query the development of EAT during Her-2 vaccination. Heterozygous C57BL/6 Her-2 transgenic mice were mated with homozygous $DR3^+$Abo mice (provided by Dr Chella David, Mayo Clinic, Rochester, MN), which expressed the HLA-DR3 transgene in the absence of endogenous mouse MHC II. The pups were either Her-2^+DR3^+ or Her-2^-DR3^+. The parental B6 Her-2 transgenic mice and the Her-2^+DR3^+ F1 mice both expressed Her-2 as a self-antigen, but with or without DR3, which dictated susceptibility to EAT. IAb of C57BL/6 mice which lack IE, mediated a resistant phenotype to EAT [32]. It has been shown in F1 mice that the parental susceptibility allele was dominant over parental resistant allele [53]. DR3 was expressed in ~20% of DR3Abo PBL and in 10–15% of F1 PBL.

Mice	C57BL/6 Her-2	X	DR3Ab^o	=	Her-2^+xDR3	+	Her-2^-xDR3
Her-2 Ag	self		—		self		—

To measure immune reactivity to Her-2 DNA vaccine, mice were electro-vaccinated with pE2TM and pGM-CSF encoding the extracellular and transmembrane domains of

Her-2 and the murine GM-CSF, respectively. To induce EAT, mice received mTg i.v. with or without LPS. Depletion of Tregs enhanced immune reactivity to Her-2 as well as mTg, verifying control of both Her-2 and mTg responses by Tregs [18]. Her-2^{+} × DR3 and Her-2^{-} × DR3 mice expressing H2b × DR3 haplotype developed more profound mTg response and thyroid pathology than Her-2 or C57BL/6 mice which expressed the EAT resistant H2b haplotype. On the contrary, Her-2 reactivity was comparable whether mice expressed HLA-DR3 or not. Therefore, induction of Her-2 immunity was independent of HLA-DR3 but development of EAT was dictated by this allele, whereas Tregs control the responses to both self-antigens.

These results, however, cannot be taken to indicate MHC independence in Her-2 reactivity. Our preliminary data with Her-2 transgenic mice in C57BL/6 versus BALB/c background suggested a significant difference in their reactivity to Her-2 DNA vaccination (not shown). Although the susceptibility to autoimmunity can be predicted, in part, by class II MHC haplotype, genetic regulation of reactivity to cancer vaccine remains to be determined.

Phase C: "clinical trials"

Her-2 DNA vaccine trials

Several Her-2 DNA vaccine trials have been initiated. A pilot clinical trial testing pVAX-E2A was conducted at the Karolinska Institute, Stockholm, Sweden, in stage IV breast cancer patients. The trial entitled "Vaccine immunization with nucleic acid coding for the gene Her-2/neu together with low doses GM-CSF (Leucomax*) and IL-2 (Proleukin*) as adjuvant in patients with metastatic breast carcinoma" showed no adverse effect. In industry sponsored trials, V930 encoding Her-2 and CEA, generated by Merck, is tested in a phase I trial "V930 First in Man (FIM) Study". The patients in this study included Stages II, III, IV breast, colon, ovarian or non-small cell lung cancer patients with detectable Her-2 and/or CEA expression (http://clinicaltrials.gov/ct/show/NCT00250419?order = 1). Bavarian Nordic's subsidiary BN ImmunoTherapeutics (BNIT) started a "Phase I trial of a fixed dose of MVA-BN-HER2 following first- or second-line chemotherapy for HER-2-positive metastatic breast cancer" with MVA-BN-HER-2, a highly attenuated non-replicating vaccinia virus, MVA-BN®, engineered to encode a modified form of the Her-2 protein. http://clinicaltrials.gov/ct/show/NCT00485277?order = 1.

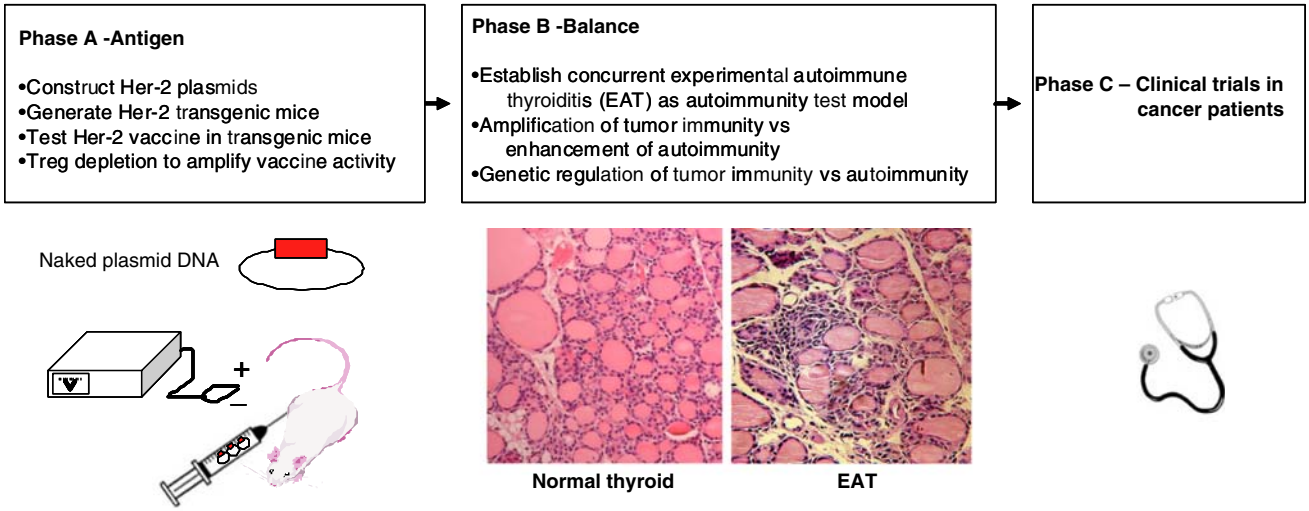
Autoimmune side effects in cancer immunotherapy trials

In cancer patients more strenuous effort than one-time Treg depletion may be required to overcome immune regulatory mechanisms before adequate vaccination efficacy can be induced, with increasing risk of autoimmunity from each new component. Our results showed that even genetically resistant BALB/c mice became susceptible to EAT after Tregs depletion, particularly during tumor regression. Patients expressing high risk HLA haplotypes will require close monitoring of their autoreactivity when their immune regulatory mechanisms are dampened. Severe autoimmune symptoms have already surfaced in cancer immunotherapy trials. In a reported clinical trial testing Her-2 peptide vaccine combined with systemic Flt-3 ligand, 2 of 15 subjects developed elevated thyroid stimulating hormone with symptoms of grade 2 hypothyroidism [39]. In another reported trial, patients with metastatic melanoma received gp100 peptide vaccines along with antagonistic mAb to CTLA-4 [48, 51, 52]. Patients with grade III/IV autoimmune manifestations were observed, including dermatitis, enterocolitis, hepatitis, hypophysitis, hypothyroidism and uveitis. The patients with objective cancer regression all developed severe autoimmune symptoms requiring intervention.

Based on the clinical experience and our findings in EAT model, patients receiving immunomodulating agents, particularly those with susceptible HLA haplotypes, should be closely monitored for autoimmune pathology. Once the importance of balancing tumor immunity with the risk of autoimmunity is recognized, novel strategies can be designed to tilt the balance toward tumor immunity. As an example, direct manipulation of the tumor microenvironment may favor immune priming in situ, thus converting the tumor into a vaccine reservoir without perturbing immune reactivity to most organ-associated self-antigens. This type of strategies to focus immunity to TAA should lead to a safer and more effective vaccine regimen.

Concluding remarks

Her-2 DNA vaccine progressed from a lab innovation to a test agent in clinical trials within a decade. With the available immune modulating tools to complement vaccination, there is optimism that Her-2 vaccine will become a reality. It will be prudent to consider potential side effect at this time, with autoimmunity as the primary concern, and to design counter measures. Although both tumor immunity and autoimmunity are immune responses to self-antigens, the pathology of autoimmunity may not equal the mechanisms of tumor rejection, thus the opportunity to dampen one without compromising the other. It may be further advantageous to deliver immune modulating agents directly into the tumor to exploit the reservoir of tumor antigens in situ, without confronting the entire immune system. Finally, the experience and insight from developing Her-2



vaccine should guide the design of the next vaccine. Surface molecules expressed in abundance and with critical functions, such as Her-2, are desirable targets, whether the molecules are expressed by tumor cells or stromal cells. With the available knowledge and technology, much will be revealed in the next decade and decisive answers to cancer vaccines can be expected.

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