

CIGB-247 vaccine breaks immunological tolerance to the vascular endothelial growth factor in mice, rats, rabbits and non-human primates, without disturbing physiologic angiogenic processes

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ABSTRACT

CIGB-247 is a novel cancer therapeutic vaccine that uses a mutated form of human vascular endothelial growth factor (VEGF) as antigen in combination with the oil-free adjuvant VSSP (very small sized proteoliposomes of *Neisseria meningitidis* outer membrane). The vaccine was designed to affect tumor neo-vascularization and tumor cell viability by eliciting antibodies that block the interaction of VEGF and its receptors in activated endothelial cells, as well as specific cytotoxic T cells that can directly destroy tumor and tumor stroma cells producing VEGF. Our previous experimental studies with CIGB-247 in mice, in which VEGF shares an 87% homology to the human molecule, have shown that the vaccine has anti-tumoral and anti-metastatic activity, and produces anti-VEGF antibodies and a specific T cell cytotoxic response against tumor cells. Herein we extend the immunogenicity and safety studies of CIGB-247 in mice, rats, rabbits and non-human primates. All the species develop antigen-specific IgG antibodies able to block the interaction of VEGF and VEGF receptor 2 in an ELISA assay. Purified IgG from CIGB-247 immunized monkey sera effectively impair human microvascular endothelial cells' proliferation and capillary-like structures formation in MatrigelTM. In monkeys and mice, DTH and direct cell cytotoxicity experiments suggest that specific T cell responses are elicited after vaccination. Immunization with CIGB-247 does not affect animal behavior, hematology counts, blood biochemistry or histology of critical organs. Skin deep wound healing was not affected in vaccinated rats and monkeys. Altogether, these results support further clinical development of CIGB-247 therapeutic cancer vaccine, and shed light on the potential mechanisms underlying its effects.

Keywords: Vascular endothelial growth factor, cancer, vaccine

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RESUMEN

La vacuna CIGB-247 rompe la tolerancia inmunológica al factor de crecimiento del endotelio vascular en ratones, ratas, conejos y primates no humanos, sin afectar los procesos angiogénicos fisiológicos. El CIGB-247 es una nueva vacuna terapéutica contra el cáncer, que emplea la forma mutada del factor de crecimiento del endotelio vascular (VEGF) como antígeno, en combinación con el adyuvante VSSP derivado de la membrana externa de *Neisseria meningitidis*. Los estudios con el CIGB-247 en ratones demostraron que la vacuna tiene una actividad antitumoral y antimetastásica, que se relaciona con una respuesta de anticuerpos anti-VEGF y de células T citotóxicas específicas. Este reporte refiere nuevas evidencias de inmunogenicidad y seguridad del CIGB-247 en ratones, ratas, conejos y primates no humanos. En estas especies, la vacuna indujo anticuerpos específicos que bloquean la interacción del VEGF con su receptor en un ensayo de tipo ELISA. Las inmunoglobulinas del tipo G (IgG) purificadas de suero de monos inmunizados afectaron la proliferación de células endoteliales microvasculares humanas y la formación de estructuras capilares en MatrigelTM. Los experimentos de hipersensibilidad retardada y de citotoxicidad directa en ratones y monos sugieren que la vacunación induce una respuesta citotóxica específica. La inmunización con el CIGB-247 resultó segura: no afectó la conducta de los animales, los conteos hematológicos, la bioquímica sanguínea ni la histología de los órganos vitales. La cicatrización de heridas profundas no se afectó en las ratas ni en los monos inmunizados. Tales resultados sustentan el futuro desarrollo clínico de esta vacuna y aportan elementos sobre los mecanismos potenciales que median su efecto antitumoral.

Palabras clave: Factor de crecimiento endotelial vascular, cáncer, vacuna

Introduction

The vascular endothelial growth factor (VEGF) and its receptors have been validated as attractive targets for developing a cancer therapeutic platform for the

treatment of different human tumors [1]. However, in cancer patients, synthetic anti-angiogenic drugs (like Sorafenib and Sunitinib), and Bevacizumab, have

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produced serious collateral side effects [2], probably due to the large doses that need to be administered. Major side effects have frequently been related to the undesired simultaneous inhibition of some physiological properties of circulating VEGF, like the induction of tissue permeability, wound healing, and maintenance of neural function [3].

We have recently developed a therapeutic cancer vaccine candidate (hereafter denominated CIGB-247) that combined the recombinant modified human VEGF produced in *Escherichia coli* as antigen, with a potent adjuvant formed by very small sized proteoliposomes (VSSP) derived from the *Neisseria meningitidis* outer membrane [4]. Experiments performed in C57Bl/6 [4] and BALB/c mice [5], challenged with four different experimental tumors, have shown that the vaccine has both anti-tumoral and anti-metastatic potential. In addition, CIGB-247 induces a transient immune response of antibodies that block VEGF-VEGF receptor 2 (KDR) interaction and generates specific cytotoxic T cells [4].

Introducing such a vaccine to the clinical practice requires further studies on the ability to break tolerance to self VEGF in species displaying higher homologies to the vaccine antigen. Also, considering the possible regulated nature of the immune response against this self-antigen, we try to probe that the vaccine could exhibit a good safety profile, different from drugs administered in bolus and exclusively focused on angiogenesis inhibition.

In this work, we offer data on CIGB-247 immunogenicity in mice, rats, rabbits, and non-human primates. Vaccination of these species consistently induces a tightly regulated humoral response, and specific IgG antibodies that exhibit VEGF-KDR interaction blocking activity. In mice and non-human primates, immunization also results in specific T-cell cytotoxic responses, measured by delayed-type hypersensitivity (DTH) and cytotoxic T lymphocyte (CTL) assays. Notably, vaccination with CIGB-247 causes no important changes in animal behavior, clinical status, blood biochemistry or histology of key organs, and allows skin deep wound healing to proceed normally in rats and monkeys [6, 7].

Results

Immunization with CIGB-247 induces specific humoral and cellular immune responses in several species

We first evaluated the effect on immune response of CIGB-247 vaccination in mice (NMRI, C57Bl/6, BALB/c), rats (Wistar and Sprague Dawley) and rabbits (New Zealand White). These experimental models go progressively to a higher homology between self VEGF and human VEGF (88.7% for mice and rats and 94% for rabbits). Our results indicated that CIGB-247 was able to induce an IgG immune response specific for VEGF in the studied species. Sera from all species impaired the binding of KDR-Fc to human VEGF as well (Table).

Additionally, vaccination of C57Bl/6 and BALB/c mice with CIGB-247 produces a specific T cell response, that can be measured through DTH and direct cell cytotoxicity against syngeneic tumor cells that produce VEGF (Table).

Table. Specific humoral and cellular immune responses in mice, rats and rabbits after CIGB-247 vaccination

Species (% of homology to hVEGF)	Strain (N)	Anti-VEGF antibody titer*	Sera blocking activity (%)	Cellular response	
				DTH	DCC
Mice (87)	BALB/c (30)	7130 ± 966	40.52 ± 3.5	+	+
	NMRI (30)	14929 ± 879	29 ± 6.0	+	nd [‡]
	C57Bl/6 (30)	3086 ± 269	46.28 ± 2.4	+	+
Rats (87)	Wistar (10)	108455 ± 1278	68 ± 5.58	nd	nd
	Sprague Dawley (8)	17904 ± 2678	31.97 ± 2.4	+	nd
Rabbits (94)	New Zealand (8)	6654 ± 400	54.59 ± 7.65	nd	nd

*IgG antibody titer vs. human VEGF in animals immunized with CIGB-247. Each column presents the average titer after the last immunization calculated from duplicate samples of individual animals and the standard deviation of the mean titers. Antibody titer and sera blocking activity were evaluated after de 6th immunization.

DTH: Delayed-type hypersensitivity.

DCC: Direct cell cytotoxicity vs. CT26 tumor cells (BALB/c mice) and vs. MB16 and 3LLD122 cells (C57Bl/6).

[‡] nd: not determined.

Evaluation of the effect of immunization schedule and adjuvant in the immune response in non human primates vaccinated with CIGB-247

Since administration schedules and adjuvant selection could significantly modulate the induction of an effective immune response to CIGB-247 nominal antigen, weekly and biweekly inoculations on VSSP and biweekly immunization adding Montanide in non human primates were evaluated. Our experiments showed that vaccination breaks B-cell tolerance to the self-growth factor (99% of homology to human VEGF) and elicits a specific and dose dependent anti-VEGF IgG response.

The weekly scheme showed a trend to higher titer values and an increased ability of the sera to block the interaction of soluble KDR-Fc with human VEGF. In this scheme, the significant increase in antibody titers after the boosters offer a clear evidence of B-cell memory (Figure A).

In general, the antibody titer kinetics in monkeys was demonstrative of a well-regulated humoral response. Anti-VEGF titers in animals immunized with CIGB-247 decline fast, and need further vaccination to be restored or augmented, in this way making it feasible to prevent any undesired persistence of anti-VEGF antibodies by simply avoiding new immunizations [6].

Antigen dose escalation study of CIGB-247 vaccine in non human primates

In this study we maintained the weekly immunization schemes in non-human primates, but increased the antigen doses in CIGB-247 from the previously tested 100 µg [6], to 200 and 400 µg. Monkeys were subcutaneously vaccinated once a week, for eight weeks. Vaccination maintenance phase started after a specific antibody titer drop was evident, and animals received three additional immunizations, once every month.

For CIGB-247, antigen dose increments produce no detectable effect in the maximum antibody titers or antibody's VEGF blocking activity. Nevertheless, higher antigen doses had a positive influence in antibody titer maintenance, after cessation of immunizations. The kinetics of titer drop after the last booster was different for each dose, with a longer retention of higher titer values for the 400 µg antigen dose

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(Figure B). These findings may prove important for the practical application of the CIGB-247 vaccine, as it could indicate that a higher antigen dose could prolong the period of time with higher circulating antibody titers, while lower doses could open the way to a tighter regulation of the antibody immune response, if desired.

Additionally, from our experiments, it is clear that boosting the immune system after the induction phase and a short resting time is relevant to achieve maximum antibody titer, antibody VEGF blocking ability and T-cell mediated responses [7].

Effect of anti-VEGF purified IgG from monkeys vaccinated with CIGB-247 on proliferation and formation of capillary-like structures in Matrigel™ of human microvascular endothelial cells

For the growth assay, human microvascular endothelial cells (HMEC) were cultured in the presence of serial dilutions of IgG purified from monkeys immunized with CIGB-247. IgG samples, Bevacizumab or negative control human IgG were diluted in serum free culture medium with or without rhVEGF (7.5 ng/mL) and added to cell cultures at a concentration range of 65-250 µg/mL. After 72 h of incubation at 37 °C in 5% CO₂-95% air atmosphere, proliferation was measured by the alamarBlue® assay (Invitrogen). Purified immune monkey IgG effectively prevent the autocrine VEGF proliferation stimulation loop of HMEC cultured cells [8], by arresting the cells in the cell-cycle G1 stage, similar to Bevacizumab. Anti-VEGF antibodies in the IgG fraction of monkey's serum act mainly as a cytostatic rather than as a cytotoxic agent *in vitro*.

To address the effect of the purified monkey IgG in the assembly of capillary-like structures by endothelial cells, HMEC were cultured in Matrigel™-coated wells and incubated with different concentrations of immunoglobulins from each antigen dose group, Bevacizumab or negative control human IgG. The number of capillary-like structures formed was quantified on an inverted microscope 16 h after incubation. Monkey IgG antibodies inhibited the ability HMEC cells to assemble into tubular vessel-like structures in the Matrigel™ assay.

These experiments indicated that, at similar total immunoglobulin concentrations, the monkey polyclonal IgG antibodies are as efficient as physiologically relevant Bevacizumab concentrations [7].

Impact of CIGB-247 vaccination on physiologic angiogenic processes

After treating many hundreds of mice in our anti-tumor protocols, as well as rats, rabbits, and non human primates [4-8], we have not found a single case where substance-related toxicity could be documented.

In particular, the studies in non human primates support our previous findings, with the outstanding feature of the relatively longer period over which the monkeys were evaluated, and the testing of larger antigen doses.

In brief, no evidence of clinical alterations in the non human primates was detected, hematological and biochemical blood parameters remain normal and

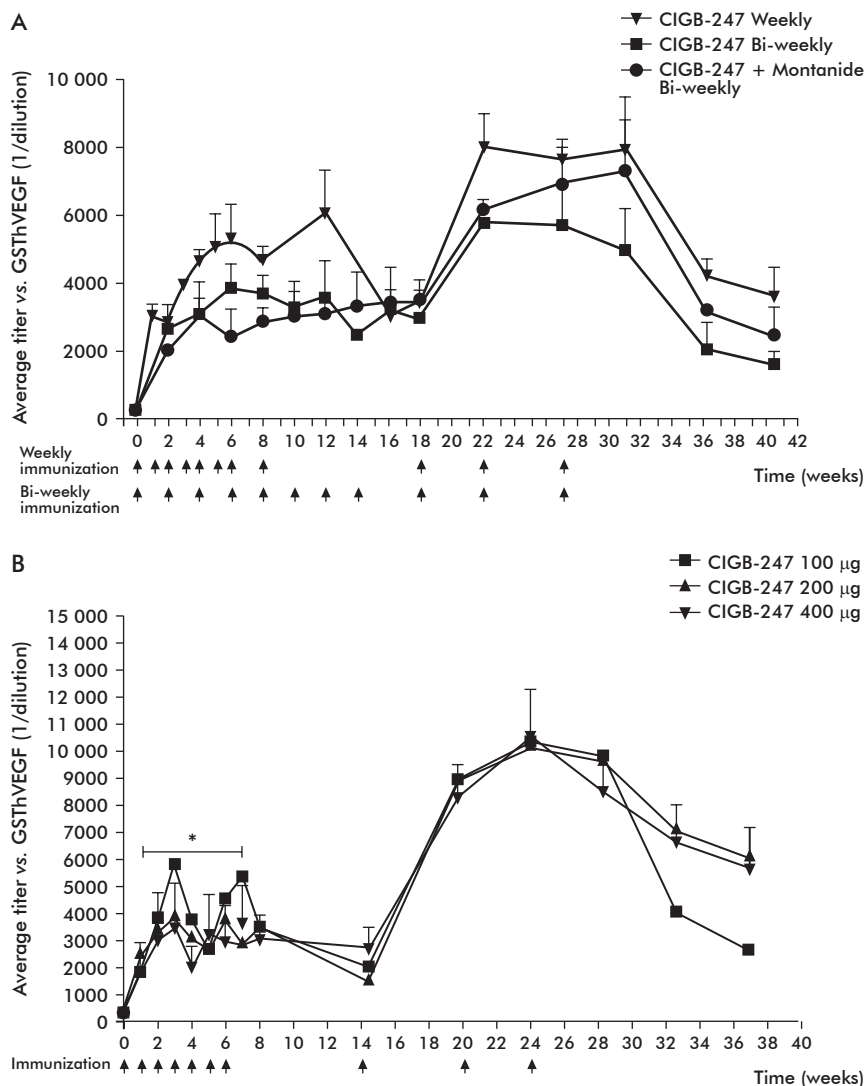


Figure. IgG antibody titer kinetics against human VEGF in monkeys immunized with CIGB-247. Each point represents the average titer calculated from duplicate samples of individual monkeys ($n = 3$) and the standard deviation of the mean titers (SEM). A) Schedule and adjuvant study (modified from [6]). Monkeys were vaccinated with CIGB-247 (100 µg of antigen), in weekly or biweekly schedules, or with CIGB-247 mixed with montanide ISA 51, in a biweekly schedule. B) Dose escalation study (modified from [7]). Monkeys were vaccinated with 100, 200 or 400 µg of antigen. * - significant differences compared to time 0 ($p < 0.05$).

blood clotting during venipuncture was unaffected, even with the highest antigen dose used for vaccination. Additionally, interference of any vaccination schedule with skin deep wound healing in rats and monkeys was not observed [6, 7].

Relevance of the study

The experiments with CIGB-247 in non-human primates showed that vaccination with this novel vaccine effectively breaks B cell tolerance to VEGF. Herein we also demonstrate that immunoglobulins purified from sera of CIGB-247 vaccinated animals, produce a biological effect in culture cells of endothelial origin that is strongly related to the possible anti-angiogenic potential of the immunization procedure. Moreover, we obtained the first evidences of the potential existence of T-cell mediated responses after

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vaccination with a VEGF-based vaccine in an animal model closely related to humans. Finally, our results also indicate that CIGB-247 vaccine could be a low toxicity alternative for cancer treatment.

Conclusions

It was demonstrated that mice, rats, rabbits, and monkeys immunized with CIGB-247 develop antibodies that block the interaction of autologous VEGF with KDR, and purified IgG from immunized monkeys are able to inhibit endothelial cell proliferation and tube-like structure formation in Matrigel™. Immunization also produces specific T-cell related responses, measured by DTH and CTL assays in different non-human primate vaccination experiments. All the evidences indicated that experimental immunization with

CIGB-247 is safe. These elements are relevant for the further clinical development of the CIGB-247 therapeutic cancer vaccine and provide clues on the potential mechanisms involved in its effect.

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