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REPORT

# Novel contributions to the study of immunological effectors induced by the specific active immunotherapy CIGB-247

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**ABSTRACT**

The primary aim of this work is to study the immunological effectors induced in CIGB-247-vaccinated patients. These effectors include humoral and cellular components of the immune system. Also, the goal of the present investigation is to detect different immunoglobulin classes and subclasses and its specificity for VEGF relevant epitopes, and demonstrate the induction of VEGF-specific and cytotoxic CD8 T lymphocytes. This research has, as an additional objective, the presentation of the first preliminary pieces of evidence of the feasibility to combine CIGB-247 with other anti-cancer treatments. To investigate the vaccine-induced immunological effectors, ELISA and ELISPOT tests were used for monitoring the immune response elicited in cancer patients from the CENTAURO and CENTAURO-2 clinical trials and also those from the compassionate use program. Immunization with CIGB-247 elicited VEGF-specific IgM and IgA antibodies. Antibody response maturation was detected in some patients with long term overall survival, indicated by the subclass switching from IgG1 to IgG4. This antibody response blocked the interaction of VEGF with its receptors VEGFR2 and VEGFR1. This specific polyclonal antibody response inhibited the binding between VEGF and the monoclonal antibody bevacizumab, for that reason, it was directed to a relevant epitope on VEGF, implicated in the VEGF-induced proangiogenic activity. The vaccination contributed to normalizing platelet VEGF levels, and produced cytotoxic CD8 T lymphocytes. Some experimental evidence indicated the potential use of CIGB-247 in combination with chemotherapy. Conclusions: All these results allowed for the application of CIGB-247 in phase II clinical trials in combination with other cancer therapeutic treatments. This work received the Annual Award of the Cuban Academy of Sciences for the year 2020.

*Keywords:* VEGF, CIGB-247, specific active immunotherapy, cancer vaccine

**RESUMEN**

**Nuevas contribuciones al estudio de los efectores inmunológicos inducidos con la inmunoterapia activa específica CIGB-247.** Esta investigación tiene como objetivo estudiar los efectores inmunológicos que se inducen en los pacientes inmunizados con el CIGB-247, que incluyen elementos humorales y celulares. Este estudio pretende detectar las diferentes clases y subclases de inmunoglobulinas, evaluar su especificidad por epítopes relevantes en el VEGF y demostrar la presencia de linfocitos CD8+ citotóxicos específicos. También, este trabajo se propone presentar las primeras evidencias preliminares de la posibilidad de combinar el CIGB-247 con otros regímenes oncoterapéuticos. Para el estudio de los efectores inmunológicos, se realizó el seguimiento inmunológico por las técnicas de ELISA y ELISPOT a los pacientes de los estudios CENTAURO, CENTAURO-2 y Programa de Uso Compasional. Se demostró que la inmunización induce anticuerpos de clase IgM e IgA específicas al VEGF. En algunos pacientes de larga supervivencia, se produjo una maduración de la respuesta de anticuerpos, representado por el cambio desde IgG1 hacia la subclase IgG4 de mayor afinidad. Se demostró que la repuesta de anticuerpos bloquea la interacción entre el VEGF y los receptores VEGFR2 y VEGFR1. Esta respuesta policlonal específica al VEGF está dirigida a un epítope relevante para su actividad pro-angiogénica, al inhibir su interacción con el anticuerpo monoclonal bevacizumab. Se muestra que la inmunización contribuye a normalizar los niveles de VEGF plaquetario y genera linfocitos T CD8+ citotóxicos. Se obtienen evidencias de la posibilidad de combinar el CIGB-247 con la quimioterapia. Conclusiones: Estos resultados permitieron el avance del CIGB-247 hacia los estudios clínicos fase II en combinación con otros regímenes onco-terapéuticos. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2020.

*Palabras clave:* VEGF, CIGB-247, inmunoterapia activa específica, vacuna para cáncer

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## Introduction

CIGB-247 is a vaccine for cancer therapy, which uses a recombinant molecule, representative of isoform 121 of the vascular endothelial growth factor (VEGF), as antigen [1]. The antigen is combined with adjuvant VSSP, of bacterial origin [2], which incorporates the N-acetyl GM3 ganglioside obtained from a natural

source. Vaccination with CIGB-247 in experimental animal models is safe [3] and has antitumor and antimetastatic effects [1, 4].

The first exposure of humans to the CIGB-247 vaccine was evaluated within the framework of the CENTAURO clinical study, in patients with advanced solid tumors. The group immunized with the highest antigen dose exhibited more favorable results for the induction of IgG class antibodies specific to VEGF, in the capacity for blocking the interaction of the VEGF molecule with VEGFR2, the significant reduction of platelet VEGF and the generation of specific and IFN-γ-secreting T-lymphocyte clones [5]. Although this group turned out to be the one with the highest advantages, from the immunological point of view, it also indicated the need for increasing the dose of antigen and adjuvant or the use of alternative adjuvants, aimed for improving immunogenicity. Consequently, the CENTAURO-2 study began [6].

In parallel, the continuity of the immunization out of the framework of the CENTAURO clinical study allowed documenting signs of objective clinical benefits in a group of patients [7]. These results laid the foundations to propose the use of CIGB-247 in a Compassionate Use Program (CUP), in patients without other therapeutic alternatives or in those who did not meet the inclusion criteria of the vaccine's clinical studies [8].

These three studies in patients allowed knowing in detail the immunological effectors that could be participating in the vaccine's mechanism of action and the potential causes producing antiangiogenic and antitumor effects. For instance, there was unknown whether, aside from IgG, the antibody response generated other immunoglobulin classes like IgM and IgA. Knowing this element is important because the participation of other classes of immunoglobulins may contribute to the ligand inhibition and to involve other biological effects mediated by their constant Fc regions.

So far, it has not been specified whether the antibody response is capable of blocking the interaction of VEGF with the other receptor, VEGFR1, even if it had the dual-blocking property, that is, inhibiting in a simultaneous manner the VEGF/VEGFR2 and VEGF/VEGFR1 interactions. This is relevant upon taking into account that both interactions mediate processes like tumor angiogenesis and tumor-induced immunosuppression [9, 10], mechanisms that tumors use for their development and dissemination. In this sense, it had not been evaluated whether polyclonal antibodies prevented the binding of VEGF to the monoclonal antibody bevacizumab. This antibody has specificity for an epitope relevant for the proangiogenic activity of VEGF, and it has been approved by the FDA for the treatment of different types of tumors [11-16].

Monoclonal antibody bevacizumab is an antiangiogenic with associated adverse effects related to the high doses administered and the abrupt effect in the reduction of VEGF [17]. Although the active immunotherapy with CIGB-247 induces a significant reduction in the platelet VEGF levels [5], there is unknown whether these levels decrease below the physiological values. Therefore, there was necessary to investigate the platelet VEGF levels in healthy individuals and compare them with the levels detected in vaccinated patients.

There are relatively few cancer-vaccine studies that include long immunization periods, hence, the information regarding the changes that take place in the immunological response generated in this type of patient is limited. Upon increasing the number of patients vaccinated with CIGB-247, there was an increased number of individuals showing higher accumulated survival times exceeding one year and even beyond three years [6-8]. This opportunity allowed studying the presence and characteristics of the immunological effectors, not described so far, which included IgG subclasses and cytotoxic T CD8+ lymphocytes.

Therefore, this work was aimed to study the immunological effectors induced in the patients immunized with CIGB-247, which include humoral and cellular elements. This study intends to detect the different classes and subclasses of immunoglobulins, evaluate their specificity for relevant VEGF epitopes and demonstrate the presence of specific cytotoxic T CD8+ lymphocytes. Likewise, this work intends to present the first preliminary evidences supporting the possibility of combining CIGB-247 with other oncotherapeutic regimes.

## Materials and methods

### Vaccine antigen and adjuvants

The antigen and the aluminum phosphate adjuvant were produced at CIGB's Development Unit, under Good Manufacturing Practices conditions. Adjuvant VSSP was produced at the Center of Molecular Immunology. The antigen was diluted in water and mixed with the adjuvant. The volume of the immunogen never exceeded 1 mL, and it was administered by the subcutaneous route.

### Selection of patients

The patients included in this research correspond to clinical studies CENTAURO (RPCEC00000102) and CENTAURO-2 (RPCEC00000155), available at: http://registroclinico.sld.cu/. There were also included patients who were recruited in a CUP, complying with all the requirements established in Regulation 63-2012, issued by the Regulating Authority of Medicaments, Equipment and Medical Devices of the Republic of Cuba.

The patients of the CENTAURO clinical study continued the monthly immunizations with the 400-µg antigen dose and 200 µg of the VSSP adjuvant. The CENTAURO-2 clinical study was a phase I-b clinical study conducted in patients with advanced solid tumors of different types [6]. CUP included oncological patients with solid or hematological tumors [8]. All the studies were carried out under the strict fulfillment of the ethical requirements established by the Declaration of Helsinki.

The CENTAURO-2 clinical study recruited a total of 50 patients, randomly distributed in five groups of ten individuals each. The experimental groups were the following: 400 µg of antigen plus 200 µg of VSSP, 400 µg of antigen plus 400 µg of VSSP, 800 µg of antigen plus 200 µg of VSSP, 200 µg of antigen plus 0.7 mg of $Al^{3+}$, 400 µg of antigen plus 0.7 mg of

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$Al^{3+}$. CUP included a total of 153 patients, who were immunized with 400 µg of antigen plus 200 µg of VSSP. The combinations of the antigen with adjuvant VSSP or aluminum phosphate were administered as previously described [6].

### Selection of apparently healthy individuals

A total of 93 apparently healthy individuals gave their consent for extracting their blood following the practice of the institution (CIGB). Patients with previous medical history of cancer, inflammatory diseases, diabetes, sicklemia or use of anti-inflammatory drugs, nor women in menstrual period, were not included.

### Obtention of peripheral-blood mononuclear cells

The peripheral-blood mononuclear cells (PBMCs) were isolated from the blood collected in tubes containing EDTA as anticoagulant and by means of a Ficoll gradient (GE Healthcare, 17-1440-03). The PBMC layer or ring was extracted and frozen in bovine fetal serum supplemented with DMSO at 10 %, in cryogenic vials that were preserved in liquid nitrogen.

### Conjugation of bevacizumab antibody to biotin

The bevacizumab antibody was covalently conjugated to the biotin molecule according to the procedure previously described [8] and using the relation of 0.1 mg of biotin per mg of antibody.

### Purification of the IgG fraction from sera of vaccinated patients

Sera obtained following the initial vaccination, classified as positive to anti-VEGF IgG antibodies and coming from different patients included in the CUP, were mixed. Sera IgG fraction was purified by affinity chromatography, according to the manufacturer's instructions, with a protein A matrix (GE Healthcare, 17-1279-02).

### Indirect ELISA for the detection of antibody classes IgG, IgM and IgA with specificity for human VEGF

The different classes of antibodies were detected as previously described [6, 18]. Briefly, the ELISA was based on immobilizing the human VEGF in a high-binding ELISA plate. Afterwards, human sera were added and each antibody class was detected with an antibody conjugate specific to each one. The reaction was developed with OPD or TMB as chromogens, and $H_2O_2$ as enzyme's substrate. The absorbance values were directly proportional to the amount of serum antibodies.

### Competitive ELISA for evaluating the capacity of human sera to block the VEGF/VEFFR2, VEGF/VEGFR1 or VEGF/bevacizumab interactions

The ELISA assay was conducted as previously described [6, 8, 18]. Human VEGF was immobilized in a high-binding ELISA plate, for evaluating the blocking capacity of sera on each of these interactions. Afterwards, sera were added and simultaneously incubated with VEGFR2, VEGFR1 or biotinylated bevacizumab. The amount of receptor bound to VEGF was detected with an antibody conjugate, and specific to each molecule. In the case of biotinylated bevacizumab, peroxidase-streptavidin conjugate was used. The absorbance values were inversely proportional to the blocking activity contained in sera.

### Indirect ELISA for the detection of IgG subclasses specific to human VEGF

A high-binding ELISA plate was coated with human VEGF, at a concentration of 2.5 µg/mL. After the blocking step, human sera were applied. The presence of each specific IgG subclass in sera was detected using anti-subclass biotinylated antibodies (anti-IgG1, anti-IgG2, anti-IgG3 or anti-IgG4, respectively) [6].

### VEGF quantification

VEGF levels were quantified using a commercial ELISA provided by R&D Systems (SVE00). The assay was conducted according to the manufacturer's instructions. Platelet VEGF was expressed in pg of VEGF/$10^6$ platelets [19].

### ELISPOT for the detection of peripheral, IFN-γ-secreting CD8+ lymphocytes specific to VEGF

The assay was conducted with peripheral-blood mononuclear cells as previously described [5, 7]. Briefly, the number of positive, alive and non-apoptotic CD3 cells was counted by flow cytometry. From 100 000 to 150 000 cells were seeded in a U-bottom plate. Then, cells were stimulated with VEGF, an anti-CD28 monoclonal antibody and IL-2, for eight days. CD8+ cells were selected by seeding of re-stimulated cells in an ELISPOT plate (R and D Systems, EL3094). The procedure was performed according to the manufacturer's instructions. IFN-γ secretion was detected with a cytokine-specific antibody. The IFN-γ-secreting clones were counted using an ELISPOT plate reader (AELVIS, Germany) and the ELI Analyse program.

## Results and discussion

### Immunization with CIGB-247 induces IgM and IgA antibodies aside from IgG, specific to VEGF

Figure 1 shows the inverse value of the antibody titers specific to VEGF, for IgG, IgM and IgA immunoglobulin classes. In the CENTAURO-2 clinical trial, 26 out of the 39 patients were positive to IgG antibodies, 11 to IgA and 7 to IgM. The group of patients vaccinated with the maximum antigen dose (2Ag + V) exhibited the highest number of positive patients to specific IgG, IgM or IgA antibodies classes (Figure 1 A-C). Regarding antibody titers, the values indicated the hierarchy: IgG > IgM > IgA (Figure 1A-C).

Figure 1D shows a similar analysis for the patients who were included in the CUP. This study confirmed the results obtained in CENTAURO-2 clinical trial, indicating IgG as the predominant immunoglobulin, also accompanied by IgM and IgA.

For the first time, it was demonstrated that the immunization induces antibodies class IgM and IgA, specific to VEGF [6, 8], and immunoglobulins that could contribute, along with IgG, to the ligand's block and to the participation of other biological processes mediated by the Fc region.

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VEGF is a soluble factor which ligand depletion is the most important effector mechanism. Therefore, IgM and IgA may block the interaction between VEGF and the receptors. Aside from the ligand depletion, the potentiation of the antigen presentation mediated by FcγR is another mechanism that contributes to the immunity against tumors [20]. It is known that immunocomplexes formed by IgG antibodies and the antigen may increase the antigen capture by dendritic cells, thereby potentiating the cross-presentation of the same [21, 22]. A similar mechanism is described for IgM- and IgA-type antibodies through Fcα/μR or FcαRI [23, 24]. This type of mechanism could be present and involve specific IgM and IgA antibodies induced with CIGB-247.

### The chronicity of vaccination induces an antibody subclass switch from IgG1 to IgG4 of higher affinity

IgG1 was the predominant subclass in all the periods of the CENTAURO-2 clinical study (Figure 2A). The presence of subclass IgG4 increased over time as the predominant immunoglobulin, from 0 % in weeks 5 to 16, to 30.8 % of patients in weeks 46 to 56. Therefore, IgG4 was the second subclass of relevance, one year following re-immunizations [6].

A similar analysis was made in the samples of patients included in the CUP (Figure 2B). In this case, the study covered until the three years of chronic vaccination. IgG1 was the predominant subclass during the induction phase and on weeks 49 and 96. In that period, subclass IgG4 occupied the second place of relevance. Between the two and three years of repeated immunizations, subclass IgG4 was the preponderant immunoglobulin since it was detected as predominant in 43 % of sera samples (Figure 2B).

In long-term surviving patients, there were evidences about the possibility to induce a change from IgG1 to subclass IgG4 of higher affinity [6, 8]. This result was also obtained in the patients of the CENTAURO clinical study, who exhibited more than three years of accumulated survival [7]. Therefore, with the chronicity of vaccination, there was not detected the presence of immunological tolerance, rather there is a maturation of the humoral immune response. For the first time, there is reported the generation of antibodies of higher affinity in the context of vaccination with CIGB-247. This characteristic is relevant for blocking VEGF as soluble factor. The induction of a specificity for VEGF by different classes of immunoglobulins and different IgG subclasses is, without any doubt, a favorable element for obtaining a neutralizing effective response against this growth factor. If there is added the polyclonal quality for each type of antibody, then the capacity of this response to inhibit VEGF or to block its interaction with the natural receptors becomes much more effective.

### Immunization with CIGB-247 induces antibodies with the capacity to block the VEGF/VEGFR2, VEGF/VEGFR1 and VEGF/bevacizumab interactions

The CENTAURO-2 clinical study confirmed the results showing that CIGB-247 induces an antibody response that block the interaction of VEGF with VEGFR2

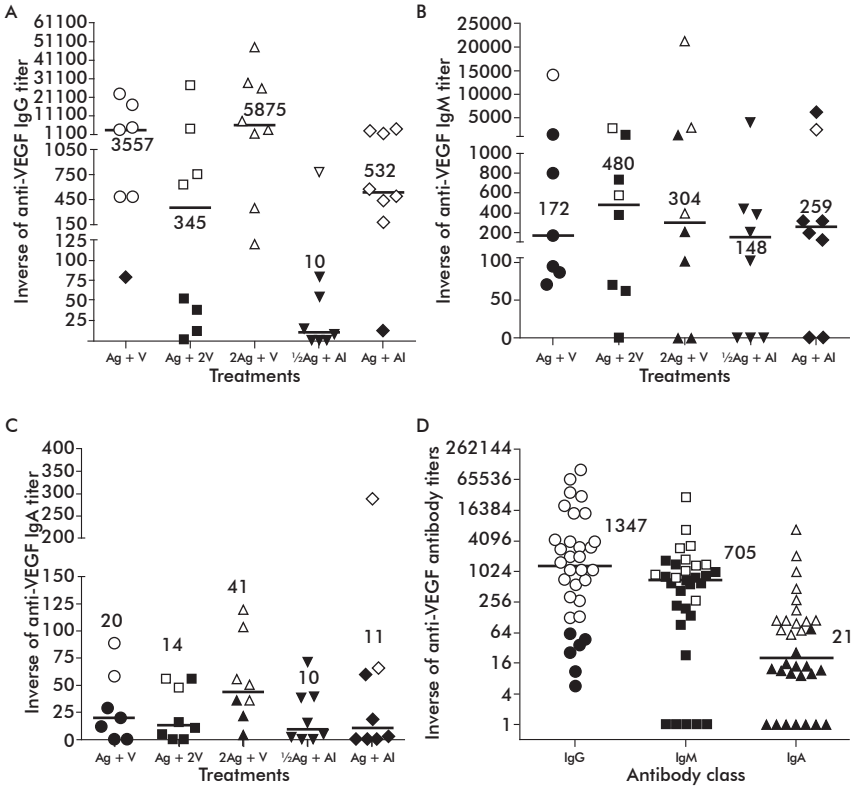


Figure 1. Antibody titers specific for human VEGF detected during the induction phase, from patients of the clinical studies CENTAURO-2 (A-C) and a Compassionate Use Program (CUP) (D). Antibody titer specific for antibody class: A) IgG. B) IgM. C) IgA. D) Antibody classes in the CUP study. The inverse value of the antibody titer corresponding to the pre-vaccination value was subtracted to the post-vaccination value; this being the result shown in the "y" axis. Horizontal bars indicate median values of the antibody titers, the values shown for each one of the groups of treatment or for each one of the antibody classes. White and black symbols represent patients with serum samples positive or negative to antibodies, respectively. Legend: Ag + V: 400 μg of antigen plus 200 μg of VSSP. Ag + 2V: 400 μg of antigen plus 400 μg of VSSP. 2Ag + V: 800 μg of antigen plus 200 μg of VSSP. ½Ag + Al: 200 μg of antigen plus 0.7 mg of $Al^{3+}$. Ag + Al: 400 μg of antigen plus 0.7 mg of $Al^{3+}$. Results published in the journal BMC Immunology [6, 8].

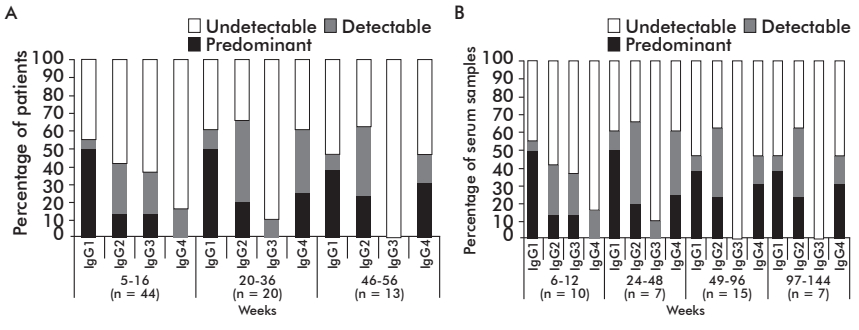


Figure 2. IgG subclasses specific to VEGF generated with the immunization during the induction and maintenance phases. A) Study in the patients of clinical study CENTAURO-2. B) Study in patients of a Compassionate Use Program. N: Number of evaluated patients. n: Number of evaluated serum samples. Results published in the journal BMC Immunology [6, 8].

(Figure 3A) [6]. Nevertheless, for the first time, both in the CENTAURO-2 study and in CUP, there was demonstrated the existence of other qualities of the humoral immune response like its dual capacity to simultaneously block the binding of VEGF to VEGFR2 and VEGFR1 (Figure 3B and C). The detection of a

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dual blocking activity indicates that CIGB-247 constitutes a valid strategy to block the VEGF/VEGFRs axis. That pathway actively participates in the stimulation of processes like tumor angiogenesis and tumor-induced immunosuppression [9, 10]. Both processes are fundamental for the development of tumors and their metastases.

The IgG fraction purified from the sera of vaccinated patients (IgG comp) indicated the presence of antibodies that block the VEGF epitope recognized by the monoclonal antibody bevacizumab (Figure 3D). This result indicated that an epitope is compromised in the VEGF, relevant for its pro-angiogenic activity. This aspect is of great interest, taking into consideration that this antibody has been approved by FDA for the treatment of several types of tumor [11, 13-16, 25].

## Immunization with CIGB-247 does not induce a complete reduction of platelet VEGF but contributes to normalizing its levels

Previous to vaccination (week 0), the platelets from cancer patients loaded more VEGF than the platelets from healthy individuals (Figure 4A). Following vaccination (week 13), there were no significant differences in the platelet VEGF levels between healthy individuals and cancer patients (Figure 4B). After vaccination, the platelet VEGF levels in the cancer patients were within the physiological range established from the measurement carried out in 93 apparently healthy individuals. These results indicated that vaccination with CIGB-247 contributes to normalize these levels [26].

This new characteristic of CIGB-247 of normalizing the platelet VEGF levels in the presence of the highest antibody levels (week 13) is a distinctive element that differs with respect to the antibody bevacizumab. For instance, Karp *et al*. proved that two hours after the intravenous administration of bevacizumab, the patients may experience a complete reduction in the VEGF bioavailability [27]. Hence, this abrupt reduction of bioavailable VEGF and caused by bevacizumab, is a totally different scenario as to the one caused by CIGB-247. Therefore, certain levels of active VEGF remain inside the platelets following vaccination, probably sufficient for sustaining vasculature and maintaining other growth factor-dependent normal physiological processes. This would also help to explain why typical toxicities reported for antiangiogenic products like bevacizumab [17] were not found in CENTAURO and CENTAURO-2 clinical trials, neither in CUP [5, 6, 8].

## Immunization with CIGB-247 generates cytotoxic CD8+ T lymphocytes specific to VEGF

The table depicts the results of the CD8+/IFN-γ ELISPOT test, performed in CENTAURO clinical trial patients, which showed a survival between two and three years. The patients exhibited a survival between two and three years. On week 49, the eight patients were positive for the IFN-γ ELISPOT assay; four of the same (CH07, CH15, CH19 and CH28) exhibited IFN-γ secreting-CD8+ lymphocytes. In two patients (CH11 and CH25), there was not detected secretion of the cytokine related to this subpopulation of T lymphocytes. Between weeks 129 and 145, four patients (CH07, CH11, CH19 and CH28) showed IFN-γ-secreting activity associated to CD8+ T lymphocytes, while in one patient (CH15), such cytokine secreting activity was not detected. Patient CH18 was classified as negative in the IFN-γ ELISPOT assay, while showing positivity for the secretion of the cytokine associated to CD8+ lymphocytes.

For the first time, it was demonstrated that the vaccination with CIGB-247 is able to maintain a

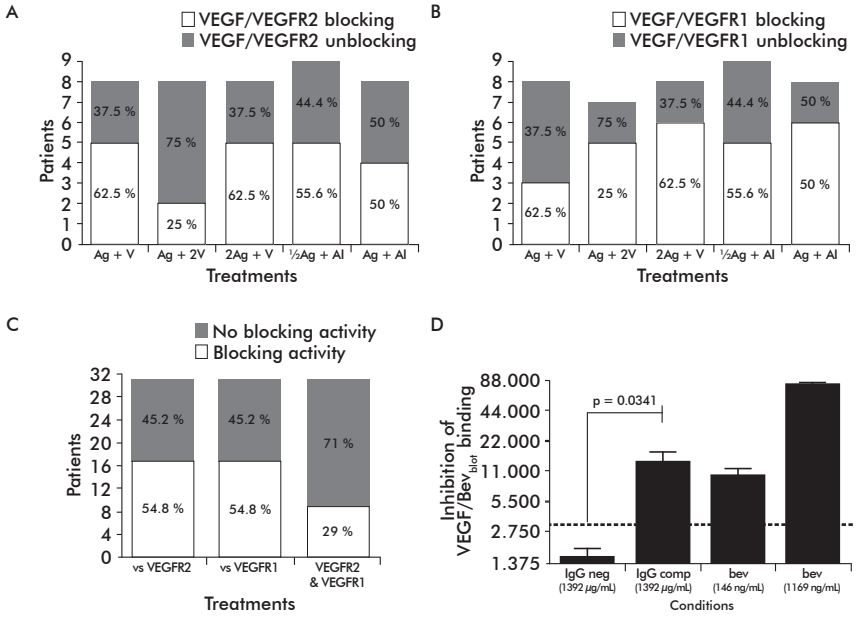


Figure 3. Blocking activity during the induction phase generated against the VEGF/VEGFR2, VEGF/VEGFR1 and VEGF/bevacizumab interactions. A) Blocking activity on the VEGF/VEGFR2 interaction. B) Blocking activity on the VEGF/VEGFR1 interactions, in the patients of clinical study CENTAURO-2. C) Blocking activity on the VEGF/VEGFR2 and VEGF/VEGFR1 interactions generated during the induction phase, in patients included in a Compassionate Use Program. D) Inhibition of the VEGF/bevacizumab interaction. The broken lines represent the cut values which define the positivity of the test. Bevacizumab was used as positive inhibition control. IgG comp and IgG neg were evaluated at the same total IgG concentration and p values were calculated according to the non-paired Student's t test. There was considered statistical significance when there was obtained a p value < 0.05. Legend: 400 µg of antigen plus 200 µg of VSSP, Ag + V; 400 µg of antigen plus 400 µg of VSSP, Ag + 2V; 800 µg plus 200 µg of VSSP, 2Ag + V; 200 µg of antigen plus 0.7mg of $Al^{3+}$, ½Ag + Al; 400 µg of antigen plus 0.7mg of $Al^{3+}$, Ag + Al. Results published in the journal BMC Immunology [6, 8].

Table. Results of positive ELISPOT CD8α/IFN-γ in patients of CENTAURO clinical study subjected to sustained vaccination with CIGB-247 for more than two years

| | Week 49 | | Weeks 129-145* | |
|---|---|---|---|---|
| Patients | IFN-γ | CD8+ IFN-γ | IFN-γ | CD8+ IFN-γ |
| CH07 | + | + | + | + |
| CH11 | + | – | + | + |
| CH15 | + | + | + | – |
| CQ17 | + | ND | – | ND |
| CH18 | + | ND | – | + |
| CH19 | + | + | + | + |
| CH25 | + | – | – | – |
| CH28 | + | + | + | + |

+: Cell sample classified as positive for the ELISPOT test. –: Cell sample classified as negative for the ELISPOT test. (IFN-γ): Measurement of interferon gamma measured in total PBMCs. CD8+ IFN-γ: Measurement of IFN-γ in the population of CD8+ lymphocytes selected from total PBMCs. ND: Not determined due to sample unavailability. *: Weeks expressed in the form of range since not all the PBMC samples were collected on the same week for different patients. Results published in magazine Vaccine [7].

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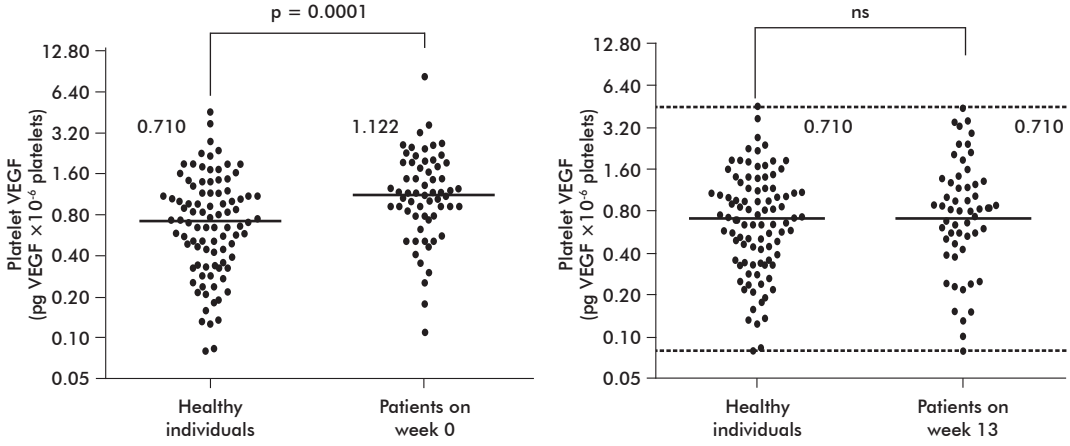


Figure 4. Platelet VEGF levels in healthy individuals and in cancer patients. Both groups of subjects included men and women. The cancer patients were 24 individuals from the CENTAURO study and 38 individuals from the CENTAURO-2 study, for a total of 62 subjects. A): Platelet VEGF levels in healthy individuals and in cancer patients before receiving the first immunization (week 0). B): Platelet VEGF levels in healthy individuals and in cancer patients after the completion of the induction phase (week 13). Each point represents one patient and the horizontal bars indicate the median of the platelet VEGF, indicating their corresponding values. The broken lines represent the range (maximum and minimum) of the platelet VEGF levels obtained from the 93 healthy individuals. The values of p parameter were calculated according to Mann-Whitney's test. There was considered statistical significance when a p value < 0.05 was obtained. ns: Non-significant. Results published in magazine BMC Immunology [26].

cellular immune response mediated by T lymphocytes specific to human VEGF, potentially represented by cytotoxic CD8+ T lymphocytes. Up to our knowledge, there is no VEGF-based vaccine that generates this type of lymphocytes in humans. These cellular elements of the immune response are relevant in the activity against tumors, given their capacity to directly eliminate malignant cells. The presence of a CD8+ T cell-mediated immune response at the different stages of the process of vaccination with CIGB-247 may explain, to a certain extent, the clinical benefits observed in a number of patients with survival exceeding two years [7].

## Immunization with CIGB-247 may be combined with other oncological therapies

CUP made possible the administration of the vaccine to cancer patients with different characteristics and under distinct treatments from those of patients included in the CENTAURO and CENTAURO-2 clinical studies [8]. This new context allowed evaluating the safety and immunogenicity of CIGB-247 in oncological patients with characteristics closer to the medical practice in Cuba. For instance, patients vaccinated with CIGB-247 and concomitantly treated with radiotherapy, chemotherapy, biological therapies or immunosuppressive drugs did not meet the inclusion criteria. In this new scenario, CIGB-247 was able of inducing a detectable response of VEGF-specific antibodies. There were also evidences of the potentiality of combining the CIGB-247 vaccine with chemotherapy, without affecting the anti-VEGF response [8]. These results laid the foundations for the design of a phase II clinical study of efficacy, currently conducted, which includes the combination of CIGB-247 with chemotherapy and surgery (RPCEC00000246).

## Relevance of the immunological effectors and proposal of a mechanism of action

CIGB-247 is the first vaccine targeting VEGF, which was administered in humans and its results are supported in two phase I clinical studies, denominated CENTAURO and CENTAURO-2, and in one CUP. These studies proved the immunogenicity of CIGB-247 in cancer patients.

The presence of different classes (IgM and IgA) and subclasses (e.g., IgG4) within the immune response, as well as the detection of specific CD8+ T lymphocytes, turn CIGB-247 into the only active immunotherapy based on human VEGF, with evidences on the activation of both, the humoral and cellular branches of the immune system.

Aside from CIGB-247, there is only one vaccine candidate, named VEGF 26-104-/RFASE, developed by Wentink and collaborators [28], which has been evaluated in the context of a phase I clinical study, in patients with advanced solid tumors. In none of the 18 patients immunized, there was detected an antibody response specific against the autologous human VEGF [29].

The analysis of all the results obtained for CIGB-247 allowed establishing a possible mechanism of action of the vaccine. It is based on the generation of a polyclonal antibody response characterized by the presence of immunoglobulins of different classes and subclasses, exhibiting different avidity and affinity for VEGF, giving way to a more effective block of its pro-angiogenic activity. Upon reducing the pro-angiogenic activity, there is a negative impact on tumor angiogenesis. Likewise, there is activated the cell-mediated immune response with the induction of cytotoxic CD8+ T lymphocytes, capable of producing tumor cell death by apoptosis. The effects provoked by the humoral and cellular

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branches of the immune response exhibit antitumor activity.

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## Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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