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Short communication

Impact on antitumor response using a new adjuvant preparation as a component of a human papillomavirus type 16 therapeutic vaccine candidate

Milaid Granadillo [a,*], Aileen Batte [a], Alain B. Alfonso [a], Aracelys Blanco [b], Dioslaida Urquiza [b], Laura Varas [c], Dayana Soler [c], Maelys Miyares [c], Miladys Limonta [c], Isis Torrens [a]

[a] Pharmaceutical Department, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Cubanacán, Playa, Havana 10600, Cuba
[b] Direction of Preclinical Investigations and Animal Experimentation, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Cubanacán, Playa, Havana 10600, Cuba
[c] Direction of Technological Development. Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Cubanacán, Playa, Havana 10600, Cuba

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ABSTRACT

Cervical cancer is a global public health problem and human papillomavirus (HPV) 16 accounts for approximately 50% of cases worldwide. Although there are several types of HPV therapeutic vaccines in clinical research, there are currently not approved for use in humans. We developed the fusion protein $LALF_{32-51}$-E7 (hereafter denominated CIGB550-E7) defined by a cell-penetrating peptide linked to an E7 mutein for the treatment of HPV16-associated tumors. We have demonstrated previously the benefit on antitumor response induced by the immunization with CIGB550-E7 admixed with very small size proteoliposomes (VSSP) adjuvant compared with the adjuvant-free immunization. In this study, we obtained a similar antitumor response in mice immunized with CIGB550-E7 admixed with the new adjuvant sVSSP that does not contain any animal-derived product. Also, the immunization with the above mentioned vaccine preparation induced a cell-mediated immune response. Our results are encouraging for the future clinical trials with the vaccine candidate CIGB550-E7+sVSSP.

1. Introduction

It is extended body of evidence that persistent infection with high-risk human papillomavirus (HPV) genotypes is the main cause of approximately all cases of cervical cancer and is also responsible for many other anogenital and oropharyngeal cancers [1]. Among females, cervical cancer ranks fourth for both incidence and mortality with an estimated 570,000 cases and 311,000 deaths in 2018 worldwide, being HPV16 and 18 are the most prevalent types associated with this disease [2].

There are currently three prophylactic HPV vaccines (Cervarix®, Gardasil® and Gardasil 9®) that have proven to be highly efficacious against persistent infection of their vaccine genotypes and associated cervical intraepithelial neoplasia [3–5]. These vaccines offer only prophylactic protection and have no demonstrated therapeutic effect for treating existing HPV infections. That́s why therapeutic vaccines are still needed to treat patients after they develop HPV-related lesions. Various types of therapeutic vaccines have been developed and tested in clinical trials and the majority target oncoproteins E6 and E7 that are the main responsible for the malignant transformation [6].

Safety profile of therapeutic vaccines represents the primary concern on their development and utilization. Thus, it is important the appropriate design of the antigen and the selection of the adjuvant. Despite the promising results from clinical trials using emerging adjuvants, there are only a few licensed for use in cancer immunotherapy, indicating the necessity to develop and characterize the novel adjuvants [7]. The very small size proteoliposomes (VSSP) adjuvant developed at the Center of Molecular Immunology [8] is a suitable adjuvant for use in cancer immunotherapy that has been evaluated in clinical trials. VSSP adjuvant is a TLR-2 and 4 agonist that promotes dendritic cell maturation, antigen cross-presentation to $CD8^+$ T cells, Th1 polarization, enhances antigen-specific cytotoxic T lymphocytes (CTL) response, protects the CTL response in immunocompromised scenarios and reduces the suppressive function of tumor-induced myeloid-derived suppressor cells [9].

We have designed and developed a "safe" fusion protein $LALF_{32-51}$-E7 (hereafter denominated CIGB550-E7) defined by a

* Corresponding author.
E-mail address: milaid.granadillo@cigb.edu.cu (M. Granadillo).

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cell-penetrating peptide linked to an HPV16 E7 mutein that has abolished its oncogenic capacity [10,11]. VSSP adjuvant has been used in combination with CIGB550-E7 in preclinical research with promissory results to treat HPV16 E7-expressing tumors [10,12,13]. The above mentioned VSSP adjuvant includes in its composition the NAcGM3 ganglioside purified from canine red blood cells [8] and this aspect could be a regulatory concern for the future clinical application of the vaccine. In order to overcome this limitation, the aim of the study was to evaluate the antitumor and cell-mediated immune response induced by the combination of CIGB550-E7 with a new preparation of VSSP that includes the NAcGM3 ganglioside obtained by chemical synthesis.

2. Materials and methods

2.1. Animal model

Female C57BL/6 mice, 6–8 weeks of age (CENPALAB, Cuba), housed under pathogen free conditions, were used in our experiments. All studies were approved by the Animal Care and Use Committee at the Center for Genetic Engineering and Biotechnology, Cuba.

TC-1 cells were obtained by co-transformations of primary C57BL/6 mouse lung epithelial cells with HPV16 E6 and E7, and an activated *ras* oncogene [14] and grown as previously described [10]. In this study, we used the more tumourigenic cell line TC-1* that was obtained as described by Granadillo et al. [12]. The cells ($5x10^4$/mouse) were administered by subcutaneous (s.c) route in the right leg. Starting 7–9 days later and every 2–3 days thereafter, the area was observed and palpated for the presence of a tumor nodule. Tumor diameters were measured in two orthogonal dimensions using electronic digital calliper (Kell-Strom, Canada) and the tumor volumes were calculated according to: $(\text{length} \times \text{width}^2)/2$ [15]. The tumor volume was evaluated until the first mouse of the experiment become moribund according to the ethics requirements.

2.2. Adjuvants and vaccine formulations

VSSP were prepared by hydrophobic conjugation of the NAcGM3 with the OMP complex of *Neisseria meningitidis*, either with the ganglioside obtained from a natural source (nVSSP) [8] or by synthesis (sVSSP). The sVSSP include as ganglioside the stearic acid. Both adjuvant preparations were provided from the Center of Molecular Immunology, Havana, Cuba.

The recombinant CIGB550-E7 fusion protein was purified as previously described [11]. We used in the experiments 120 μg of CIGB550-E7 per mouse either alone or admixed with 200 μg of nVSSP (CIGB550-E7+VSSP) or with 100 μg, 200 μg or 400 μg of sVSSP (CIGB550-E7+sVSSP). The excipients of CIGB550-E7 protein were used in the control groups alone or admixed with adjuvants.

2.3. Immunization schedule

All the immunizations were conducted in a therapeutic setting, after the tumor challenge. The immunizations started when the 100% of mice developed s.c tumors in the experiments in which they received only four immunizations. Due to we are using a more tumourigenic cell line and the tumors grow faster and the mice become moribund earlier, in the experiments of more than four immunizations (six and eight) the treatment started one day after challenge. The vaccine formulations were administered weekly by s.c route in the right flank.

2.4. Evaluation of the CTL response

The immunizations started when the 100% of mice developed s.c tumors and 7 days after the last immunization, the spleen of each mouse was processed individually and the concentration of $CD3^+$ T cells (effectors) determined in a Cyflow Space flow cytometer (Partec, Germany) using the rat anti-mouse CD3:RPE (Serotec, EUA) and following the manufacturer's instructions. The cytotoxic T lymphocytes (CTL) experiment was done as previously described [10].

2.5. Statistical analysis

All statistical analysis was performed using SPSS v.18.0 (IBM Corp., Armonk, NY, USA). The normal distribution of the data sets was analyzed using Shapiro-Wilk test and the homogeneity of variances was analyzed with Levene's test. According to the results of the above mentioned analysis, statistical significances of differences among groups were analyzed by one-way ANOVA followed by Tukey post-test for multiple comparisons or by Kruskal-Wallis followed by Dunn post-test for multiple comparisons. Differences in survival were analyzed using the Kaplan–Meier method, and groups were compared using the Log-Rank test. Values of $p < 0.05$ were considered significant.

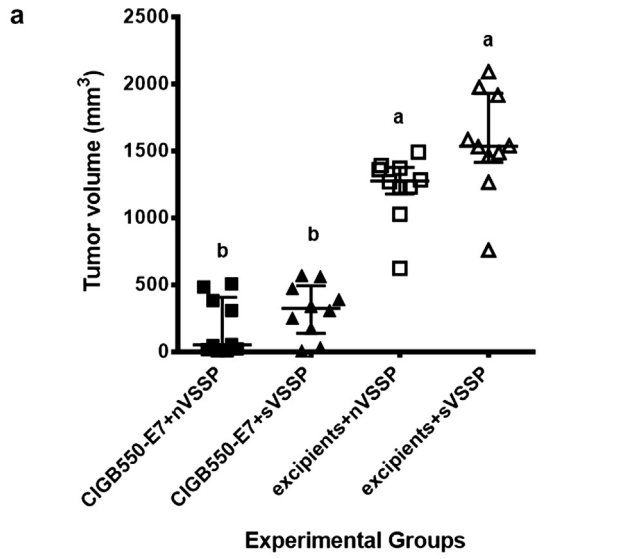
3. Results and discussion

3.1. The combination of CIGB550-E7 with sVSSP induces an antitumor response similar to that induced by its combination with nVSSP

With the aim to have a vaccine preparation that do not contain any component from animal source and that complies with the regulatory requirements for its later evaluation in clinical trials, we investigated if the combination of CIGB550-E7 with 200 μg of sVSSP adjuvant could induce an antitumor response similar to that induced with its combination with nVSSP adjuvant. C57BL/6 mice (10 per group) were challenged with TC-1* cells and when developed palpable s.c tumors they were treated with CIGB550-E7 +sVSSP, CIGB550-E7+nVSSP, excipients+sVSSP and excipients +nVSSP. The mice received four immunizations. As shown in Fig. 1A, the sVSSP as adjuvant of the CIGB550-E7 protein was able to induce an antitumor response similar to that generated by nVSSP adjuvant ($p > 0.05$; Dunn's test). In agreement with this finding, the survival (Fig. 1B) of the groups of mice immunized with CIGB550-E7+sVSSP and with CIGB550-E7+nVSSP was similar ($p = 0.7187$; Log-Rank test). The accumulated evidence obtained from several preclinical and clinical trials suggested that cancer vaccines need better adjuvants than those that are currently licensed [7], and VSSP adjuvant is a suitable candidate [9]. These are the first results that demonstrate the efficacy of the immunization with CIGB550-E7 combined with sVSSP adjuvant. Also, these results are a promising starting point for this adjuvant that recapitulates *in vivo* the effect widely demonstrated for the nVSSP adjuvant specifically designed to be formulated with vaccines used in the treatment of immunocompromised patients [9].

3.2. The combination of CIGB550-E7 with sVSSP induces a T cell-mediated immune response

It is well known that the control of HPV-associated tumors requires the generation of cell-mediated immunity, particularly T cell-mediated immunity. In this study we also evaluate the T cell-mediated immune response induced by the immunization with CIGB550-E7+sVSSP in mice with tumors. As shown in Fig. 2, the immunization with CIGB550-E7+sVSSP is able to induce a



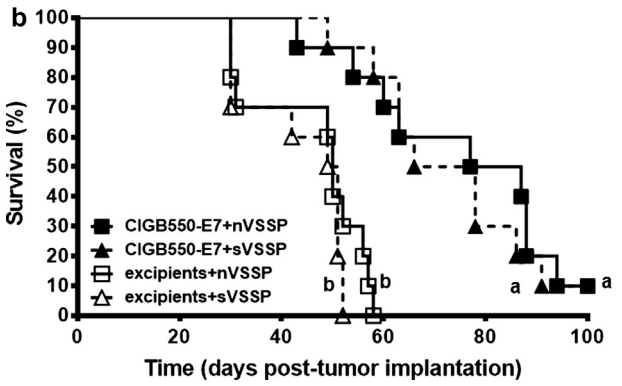


Fig. 1. Comparison of the antitumor response induced by the immunization with CIGB550-E7+sVSSP and CIGB550-E7+nVSSP. (A) Mice (10 per group) were challenged with TC-1* cells and the immunizations started when the 100% of mice developed palpable tumors. They received four immunizations at seven days intervals and tumor volumes were assessed 30 days post-implantation. The antitumor response induced by the immunization with CIGB550-E7+sVSSP was similar to that induced by the immunization with CIGB550-E7+nVSSP ($p > 0.05$; Dunn's test). The data are expressed as individual tumor volumes and median per group ± interquartile range. (B) Time to death is plotted on a Kaplan-Meier survival curve. The survival of the groups of mice immunized with CIGB550-E7+sVSSP and with CIGB550-E7+nVSSP was similar ($p = 0.7187$; Log-Rank test). Different letters indicate statistical differences among groups. Excipients in the Figure are referred to the components of the buffer (10 mM Tris-HCl, 0.01% Tween 80, pH 8.0) in which we obtain the protein CIGB550-E7.

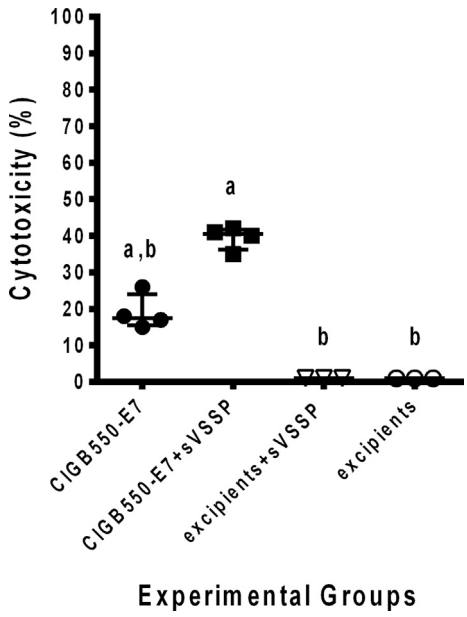


Fig. 2. Evaluation of T-cell mediated immune response. Mice (3 or 4 mice per group) were challenged with TC-1* cells and the immunizations started when the 100% of mice developed palpable tumors. They received four immunizations at seven days intervals and the CTL response was evaluated. The immunization with CIGB550-E7+sVSSP induced a CTL response. The data are expressed as individual cytotoxicity percentage and median per group ± interquartile range. Different letters indicate statistical differences among groups according to Dunn's test. Excipients in the Figure are referred to the components of the buffer (10 mM Tris-HCl, 0.01% Tween 80, pH 8.0) in which we obtain the protein CIGB550-E7.

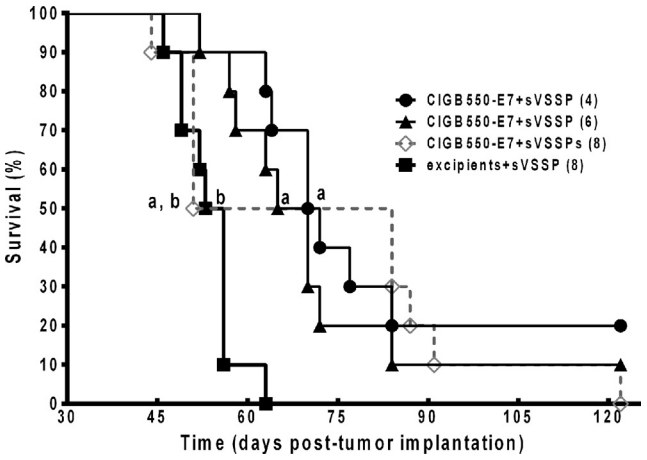


Fig. 3. Effect of the number of immunizations with CIGB550-E7+sVSSP on the antitumor response. Mice (10 per group) were challenged with TC-1* cells and the day after started the immunizations. They received four, six or eight immunizations with CIGB550-E7+sVSSP at seven days intervals and the dose of sVSSP was 200 μg/mouse. Four to six immunizations with CIGB550-E7+sVSSP were enough to improve the antitumor response with respect to the control group ($p < 0.001$; Log-Rank test). Different letters indicate statistical differences among groups. Excipients in the Figure are referred to the components of the buffer (10 mM Tris-HCl, 0.01% Tween 80, pH 8.0) in which we obtain the protein CIGB550-E7.

CTL response in agreement with the antitumor response induced *in vivo*.

3.3. Four to six immunizations and 100 μg of sVSSP per dose combined with CIGB550-E7 protein are enough to induce a potent antitumor response

The effect of both the number of immunization and the dose of sVSSP on the antitumor response was investigated. C57BL/6 mice (10 per group) were challenged with TC-1* cells and the day after treated with CIGB550-E7+sVSSP and excipients+sVSSP. The mice received four, six or eight immunizations and the dose of sVSSP used was 200 μg/mouse. As shown in Fig. 3, four to six immunizations with CIGB550-E7+sVSSP were enough to improve the antitumor response compared with the control group ($p < 0.001$; Log-Rank test). Based on this result, a similar experiment was performed, but the mice received six immunizations. The doses of sVSSP used were 100, 200 and 400 μg. The results obtained showed (Fig. 4) that increasing the dose of sVSSP did not significantly improve the antitumor response ($p > 0.05$; Tukey's test). The novel adjuvant preparation sVSSP could has a superior regulatory standard for the human use compared with nVSSP because does not

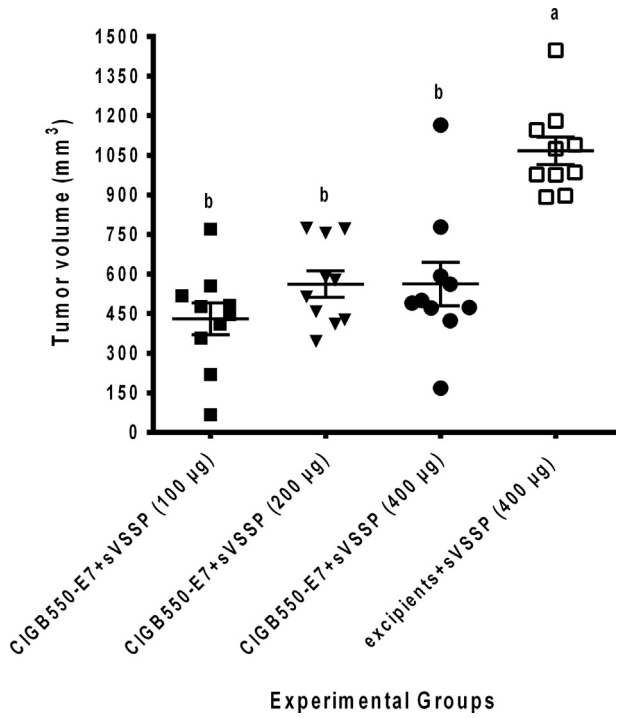


Fig. 4. Effect of the dose of sVSSP on the antitumor response. Mice (10 per group) were challenged with TC-1* cells and the day after they received the first immunization. The mice were vaccinated six times with CIGB550-E7+sVSSP at seven days intervals and the doses of sVSSP evaluated were 100, 200 and 400 µg / mouse. Tumor volumes were assessed 33 days post-implantation. The dose of 100 µg of sVSSP adjuvant was enough to induce an antitumor response similar to that generated with 200 or 400 µg ($p > 0.05$; Tukey's test). The data are expressed as individual tumor volumes and mean per group ± SEM. Different letters indicate statistical differences among groups. Excipients in the Figure are referred to the components of the buffer (10 mM Tris-HCl, 0.01% Tween 80, pH 8.0) in which we obtain the protein CIGB550-E7.

contain any animal products. Altogether, the results obtained by us will be an important guide about the doses and immunization schedule for the design of the first clinical trial with CIGB550-E7 +sVSSP.

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Conflict of interest

None.

Contribution

MG, IT and ABA were involved in the conception and design of the study. AB, DU, LV, DS, MM and ML were involved in the acquisition of data. All authors contributed to study interpretation of the data, and critical review of the paper for important intellectual content.

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References

[1] Bosch FX, Broker TR, Forman D, Moscicki AB, Gillison ML, Doorbar J, et al. Comprehensive control of human papillomavirus infections and related diseases. Vaccine 2013;31:H1–H31.
[2] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018 Sep 12.
[3] Dillner J, Kjaer SK, Wheeler CM, Sigurdsson K, Iversen OE, Hernandez-Avila M, et al. Four year efficacy of prophylactic human papillomavirus quadrivalent vaccine against low grade cervical, vulvar, and vaginal intraepithelial neoplasia and anogenital warts: randomised controlled trial. BMJ 2010;341:c3493.
[4] Wheeler CM, Castellsague X, Garland SM, Szarewski A, Paavonen J, Naud P, et al. Cross-protective efficacy of HPV-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by non-vaccine oncogenic HPV types: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial. Lancet Oncol 2012;13:100–10.
[5] Huh WK, Joura EA, Giuliano AR, Iversen OE, de Andrade RP, Ault KA, et al. Final efficacy, immunogenicity, and safety analyses of a nine-valent human papillomavirus vaccine in women aged 16–26 years: a randomised, double-blind trial. Lancet 2017;390:2143–59.
[6] Chabeda A, Yanez RJR, Lamprecht R, Meyers AE, Rybicki EP, Hitzeroth II. Therapeutic vaccines for high-risk HPV-associated diseases. Papillomavirus Res 2018;5:46–58.
[7] Temizoz B, Kuroda E, Ishii KJ. Vaccine adjuvants as potential cancer immunotherapeutics. Int Immunol 2016;28:329–38.
[8] Estevez F, Carr A, Solorzano L, Valiente O, Mesa C, Barroso O, et al. Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP). Vaccine 1999;18:190–7.
[9] Fernandez A, Oliver L, Alvarez R, Fernández LE, Mesa C. GM3-containing nanoparticles in immunosuppressed hosts: effect on myeloid-derived suppressor cells. World J Immunol 2014;4:98–106.
[10] Granadillo M, Vallespi MG, Batte A, Mendoza O, Soria Y, Lugo VM, et al. A novel fusion protein-based vaccine comprising a cell penetrating and immunostimulatory peptide linked to human papillomavirus (HPV) type 16 E7 antigen generates potent immunologic and anti-tumor responses in mice. Vaccine 2011;29:920–30.
[11] Granadillo M, Batte A, Lugo VM, Musacchio A, Bequet-Romero M, Betancourt L, et al. Expression, purification and characterization of a recombinant fusion protein based on the human papillomavirus-16 E7 antigen. Springerplus 2013;2:12.
[12] Granadillo M, Alfonso AB, Vallespi MG, Batte A, Soria Y, Brown E, et al. The $LALF_{32-51}$ peptide as component of HPV therapeutic vaccine circumvents the alum-mediated inhibition of IL-12 and promotes a Th1 response. J Cell Immunotherapy 2016;2:44–51.
[13] Granadillo M, Batte A, Blanco A, Alfonso AB, Suárez J, Merino N, et al. $LALF_{32-51}$-E7 therapeutic vaccine induces antitumor immunity against human papillomavirus type 16 E7-expressing murine tumor metastases in the lungs. Clin Exp Metastasis 2017;34:241–9.
[14] Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 1996;56:21–6.
[15] Naito S, von Eschenbach AC, Giavazzi R, Fidler IJ. Growth and metastasis of tumor cells isolated from a human renal cell carcinoma implanted into different organs of nude mice. Cancer Res 1986;46:4109–15.