

VSSP: A new adjuvant for the vaccination of immuno-compromised patients

✉ Circe Mesa, Joel de León, Luis E Fernández

Department of Vaccines, Center of Molecular Immunology
Ave 216 corner 15, Atabey, Playa, PO Box 16 040, Havana, Cuba
E-mail: circe@ict.cim.sld.cu

ABSTRACT

In the Center for Molecular Immunology, a new adjuvant was designed and described. It was created to be used in vaccines for the treatment of immuno-compromised patients. Those very small sized proteoliposomes, called VSSP, combine in their structure the GM3 ganglioside, with immune system suppressing properties and outer membrane vesicles of *Neisseria meningitidis* with demonstrated immunostimulating characteristics. This paper demonstrates that VSSPs, independently of the predominating ganglioside in their structure, provide the signals of "danger" necessary for the activation of the dendritic cells in the immune system. The authors assess how VSSPs, in their interaction with those cells, induce the secretion of inflammatory cytokines, such as IL-12, that induce the polarization to Th1 of the helper T cells. Also, it was assessed that the VSSPs, in their interaction with those cells, induce the secretion of inflammatory cytokines, such as IL-12, that induce the polarization to Th1 of the helper T cells. It was also demonstrated that the VSSPs stimulate the functional activity of the specific cytotoxic T cells, a phenomenon that is facilitated by the cross presentation of exogenous antigens and the independence of the cooperation of the helper T cells for the primary expansion of the cytotoxic T cells. Those properties were also validated by experiments that demonstrate the anti-tumor activity of two VSSP adjuvated cell vaccines.

Introduction

The adjuvants are products that increase or modulate the immune response specific for an antigen (Ag) and therefore, they are indispensable for the formulation of most vaccines. Because of that, for more than three decades endeavors and resources have been spent in scientific research for the development of new and more powerful adjuvants. Emerging theories and knowledge about the immune system regulation have strongly influenced in adjuvant development. That is the case of the theories by P. Matzinger and C. Janeway, who say that the immune system discriminates between what is dangerous and what is innocuous, through the non clone receptors that recognize molecular patterns associated to those "dangerous" signals [1, 2]. Based on those theories, new pathogens derived adjuvants have been described. These adjuvants have the peculiarity of triggering the immune response through the activation of dendritic cells (DC) and their conditioning for regulating the response of the adaptive immune system cells, mainly of the T lymphocytes. A particular aspect in the field of adjuvants research is the development of therapeutic vaccines for the treatment of immuno-compromised patients. That is the case of the vaccines for the prevention of cancer or viral chronic diseases, such as AIDS, in those cases, the tumors or viruses, interacting with the host, create mechanisms for suppressing the immune system in order to evade the vaccines action [3].

The gangliosides are glycosphingolipids that are over expressed in the tumor cells membranes and shed by them in considerable amounts as monomers, micelles and membrane vesicles. The gangliosides are powerful stimulators of the *in vivo* tumor growth and they have inhibiting ability over multiple events of the cell immune response. All those elements support the hypothesis that the gangliosides are one of the most important soluble factors in immunosuppres-

sion induced by the tumors [4]. Those immunosuppression mechanisms are multiple and are not completely elucidated. Nevertheless, it is clear that both the Ag presenting cells (APC) and the B and T lymphocytes are considerably affected by the tumor-derived gangliosides [5].

The Center of Molecular Immunology (CMI), in its endeavors for finding effective treatments against cancer, has designed an adjuvant or immuno-potentiator, with the purpose of potentiating the specific Ag response, and also interfering in the immunosuppression induced by the tumors [6]. To do that, the immunostimulating properties of a bacterial system, in this case, the outer membrane vesicles (OMV) of *Neisseria meningitidis*, with the GM3 ganglioside immunosuppressing properties were used. This combination originates very small size proteoliposome (VSSP) where the ganglioside-protein molecular ratio was 37:1. From the ganglioside molecular superiority on the *Neisseria meningitidis* proteins, at first the authors researched if the adjuvant and immuno-stimulating properties predominate in the VSSP.

Materials and methods

Mouse bone marrow dendritic cells preparation

The murine dendritic cells were obtained from precursors isolated from bone marrow from femur and tibia of just sacrificed mice. Those cells were cultured at 6×10^5 cells/mL density in D'MEM supplemented with 20 ng/mL GM-CSF (R&D, UK). A GM-CSF-supplemented culture medium was added after 72-90 hours of culture. The medium was replaced with a fresh one after eight days of culture; the DC was exposed for 18 hours to the different stimuli and their phenotype was analyzed by FACS.

1. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* 1994;12:991-1045.

2. Janeway CA Jr, et al. Cross-linking and conformational change in T-cell receptors: role in activation and in repertoire selection. *Cold Spring Harb Symp Quant Biol* 1989;54(2):657-66.

3. Pawelec G. Tumour escape: anti-tumour effectors too much of a good thing. *Cancer Immunol Immunother* 2004; 53(3):262-74.

4. Ladisch S, Kitada S, Hays EF. Gangliosides shed by tumor cells enhance tumor formation in mice. *J Clin Invest* 1987;79(6):1879-82.

5. McKallip R, Li R, Ladisch S. Tumor gangliosides inhibit the tumor-specific immune response. *J Immunol* 1999; 163(7): 3718-26.

6. Estevez F et al. Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP). *Vaccine* 1999;18(1-2):190-7.

In vivo cytotoxicity assay

Total splenocytes from naive syngenic mice were marked with CFSE flourophore (Molecular Probes, Paisley, UK) 1 μ M and SIINFEKL 1 μ M (CFSE++) or with only CFSE 100 nM (CFSE+) to obtain the target cells. For the cytotoxicity assay, 60 x 10⁶ cells of the mixture were injected into the tail vein, in the mice previously immunized with 1 mg OVA alone or mixed with 200 μ g VSSP. Sixteen hours later, the number of CFSE+ and CFSE++ cells in the inguinal ganglion closest to the immunization site was measured. The specific lysis rate was determined through the formula:

$$100 - \frac{\text{CFSE}^{++}}{\text{CFSE}^{+}} \cdot 100$$

Vaccination with irradiated cells and tumor challenge assay

The tumor model used was the CT26 colon carcinoma. In this assay, Balb/c mice were subcutaneously immunized in the left side with PBS or 10⁶ irradiated cells (75 Gy) alone or in the presence of 200 mg VSSP. Ten days after vaccination 10⁵ CT26 viable cells were also subcutaneously injected in the right side. The animals were monitored during 60 days and the tumors' growth was assessed.

Results and discussion

Dendritic cells activation

The DCs are the most frequent Ag presenting cells and play an important role in the initiation of the immune response. Thus, we evaluated the effect of VSSP in DC expression of class II MHC and a variety of co-stimulatory molecules. These experiments demonstrated that the VSSP-treated murine DCs have a high expression of the CD80, CD86, CD40 and MHC II molecules. They also demonstrated that the effect of the VSSP on the DC is a stimulus as powerful as LPS in the induction of the maturation of those cells (Table 1) [7]. This group of results was surprising because there are reports indicating that the GM3 ganglioside, highly represented in VSSP, inhibits the maturation of DC. This immunosuppressing property of the ganglioside was thwarted when combined with VSSP.

T helper cells response polarization to Th1

According to the theory proposed by Janeway about the instructive role of the innate immune system on the cells of the adaptative immune system, it has been proposed that the pattern recognition receptors expressed in the DCs interact with their ligands and, subsequently, condition the polarization of the CD4

T cells to Th1 or Th2. IL-12 has been characterized as the cytokine secreted by the DCs that condition a polarization of the helper T cells to Th1. In order to assess the VSSP ability of inducing the production of IL-12 by the DCs, the cytokine intracellular levels by flow cytometry were assessed. In this experiment, differences between LPS- and VSSP-treated DC were observed. Although a high percentage (87%) of IL-12p40/p70 producing DC were detected after stimulating with VSSP, only 34% of LPS stimulated DC produced this cytokine (Table 1) [7].

To assess the ability of the VSSP activated DC to polarize naive T CD4 lymphocytes to Th1 or Th2, those cells were purified from the DO.11.10 transgenic mice spleen. CD4 T cells from this mouse are specific for an OVA characterized peptide. In this experiment, the VSSP-activated DC promoted the secretion of IL-2 and INF γ , while the IL-4 was inhibited (non showed data). This profile of cytokines in the helper T cells is characteristic of a Th1.

Cytotoxic T lymphocytes activation

The potentiating effect of VSSP on the DC and the Th1 response conditioning, in spite of the ganglioside representativeness in the VSSP, led to the design of several experiments to determine the stimulating ability of cytotoxic CD8 T cells (CTL, cytotoxic T lymphocytes). This property had not been described before for VME of *Neisseria meningitidis*, what was a challenge for VSSP. Moreover, in the last years many stimuli have been assessed in order to know if the CD4 helper T cells are required for generating effective CTL responses. Those studies have revealed the existence of CTL responses dependent and independent on the cooperation of the CD4 cells. For assessing both phenomena with our adjuvation system, the *in vivo* CTL assay was used after a dose of OVA alone or adjuvated with VSSP and the specific response to SIINFEKL immunodominant peptide was measured. In order to find if the CD8 T lymphocytes activation occurred in absence of the helper T cells, the CTL response was measured in mice for which those cells had been removed. The removal of the CD4 T cells was performed by a systemic treatment with a specific AcM for that molecule and PBS as negative control. That experiment showed that VSSP is able to induce even 40% of the lysis in the SIINFEKL positive target cells, even in absence of the CD4 cells (Table 2) [8]. This result includes VSSP in the group of adjuvants activating the CTL response to exogenous Ag in absence of the T helper lymphocytes.

Antitumor activity

Finally, the practical assessment of the adjuvant ability of VSSP was tested in an antitumor experiment. To do that, an antigenic system different from those assessed was used. The tumor cells vaccines have been

Table 1. Activation of the murine dendritic cells.

	CD80	CD86	CD40	MHC II	IL12 p40/p70
DC ⁰	34.2	34.3	61.3	29.8	0
DC ^{LPS}	70.7	66.8	72.5	72.5	34.1
DC ^{VSSP}	67.7	62.7	70.1	69.9	86.9

* The data are shown as per cent of the positive cells to the assessed markers

Table 2. Activation of the CD8 T lymphocytes.

Vaccine	Treatment	SIINFEKL specific lysis (%)
OVA	PBS	2.4
	α CD4	2.0
OVA/VSSP	PBS	42.8
	α CD4	46.2

7. Mesa C, et al. Very small size proteoliposomes derived from *Neisseria meningitidis*: an effective adjuvant for Th1 induction and dendritic cell activation. Vaccine 2004;22(23-24):3045-52.

8. Mesa C, Fernández LE. Challenges facing adjuvants for cancer immunotherapy. Immunol Cell Biol 2004;82(6): 644-50.

widely researched since it is assumed that they content the entire relevant tumor Ags. In this study, the tumor line of CT26 colon carcinoma was one of those used for assessment. The results in the vaccination experiments with that irradiated tumor line and challenge with the live cell itself showed that the use of VSSP for adjuvating the cell vaccine prevented the development of tumors in more than 80% of the immunized animals. This result corroborates that the VSSP facilitates and increases the natural cross presentation of the CT26 apoptotic cells and induces the stimulation of specific CD8 T cells (Table 3).

The group of the results compiled in this paper demonstrates that VSSP, independently of the predominance of the ganglioside in its structure, provides the SI with the signals of “danger” necessities for the

Table 3. Antitumor activity.

Vaccine	Animals with tumor/ Total
PBS	10/10
CT26*	9/10
CT26/VSSP	1/6

activation of DCs and the polarization to Th1. Moreover, this concept is widened after determining that the VSSP signals activate and expand Ag specific CD8 T cells. This phenomenon is facilitated by the cross presentation of exogenous Ag and by the independence of the cooperation of the CD4 cells for the primary expansion of CD8. Therefore, the VSSP is a good alternative of the existing adjuvants for their use in future therapeutic vaccines.