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The $LALF_{32-51}$ peptide as component of HPV therapeutic vaccine circumvents the alum-mediated inhibition of IL-12 and promotes a Th1 response

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Abstract

Aluminum-containing adjuvants (alum) continue to be the most widely used adjuvants. It is common knowledge that these adjuvants predominantly induce humoral immunity, but some reports also describe their role combined with IL-12 in the induction of a Th1 immune response. In this study we want to investigate if alum could be an adjuvant to be used as a component of a therapeutic vaccine that require the generation of cell-mediated immunity. To demonstrate this concept we selected the human papillomavirus (HPV) 16-transformed mouse TC-1 cells as model and the fusion protein $LALF_{32-51}$-E7 as antigen. Our results suggest that $LALF_{32-51}$-E7 combined with aluminum hydroxide adjuvant promotes a Th1 immune response and consequently an anti-tumor response in the TC-1 tumor model. These results could have important application in future clinical trials in women with low grade squamous intraepithelial neoplasia.

Keywords: Adjuvant; Alum; $Al(OH)_3$; Fusion protein; $LALF_{32-51}$-E7

1. Introduction

For over 60 years, aluminum salts (alum) were the only adjuvants approved for practical vaccination by the Food and Drug Administration [1] and have been extensively used in the prophylactic vaccination. Many researchers described that alum is a very effective adjuvant for promoting humoral immunity and Th2 type T-cell responses, but it is not an effective adjuvant for inducing Th1 immune responses [2,3]. The reasons behind the poor effectiveness of alum in promoting Th1 responses are not fully understood but may include the promotion of IL-4 secreting granulocytes [3] and the inhibition of IL-12 production by dendritic cells [4]. Other studies have shown that alum combined with IL-12 can facilitate the induction of Th1 immune response [5,6]. Despite the realized efforts, it is surprising that yet is no consensus regarding the mechanisms by which alum potentiate the immune system.

Whilst prophylactic vaccination aims to the induction of antibody response, the therapeutic vaccination needs the generation of a potent cell-mediated immune response. In this study we want to investigate if aluminum hydroxide [$Al(OH)_3$] adjuvant could promote a cell-mediated immune response when is combined with an antigen. To prove this concept we

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selected a human papillomavirus (HPV) type 16 associated tumor model in mice, particularly the TC-1 model, because these tumor cells strongly express MHC class I and constitutes a suitable model to evaluate therapies that require the generation of cell-mediated immunity, particularly T cell-mediated immunity [7]. The selected antigen for this study was the $LALF_{32-51}$-E7. The $LALF_{32-51}$-E7 is a fusion protein comprising a cell penetrating peptide and HPV-16 E7 mutein that was designed to be an antigen of a protein-based therapeutic vaccine against HPV-16 induced lesions [8]. In this study we demonstrated that $LALF_{32-51}$-E7 combined with aluminum hydroxide adjuvant promotes a Th1 immune response and consequently an anti-tumor response in the TC-1 tumor model.

2. Materials and methods

2.1. Proteins and peptides

The recombinant HPV-16 E7 protein and $LALF_{32-51}$-E7 fusion protein were purified as previously described [8,9]. The endotoxin content in the final protein preparations was less than 0.05 EU/μg as measured by the chromogenic *Limulus* ameobocyte lysate assay (Lonza, USA).

The HPV-16 $E7_{49-57}$ peptide (RAHYNIVTF), a defined H-$2D^b$-restricted cytotoxic T lymphocyte (CTL) epitope [10]; the HPV-16 $E7_{48-57}$ peptide (DRAHYNIVTF), a defined CD4 T cell epitope [11] and the $LALF_{32-51}$ (HYRIKPTFRRLKWKYKGKFW) from the *Limulus* anti-lipopolysaccharide factor, were synthesized and purified as previously described [8].

2.2. Adjuvants

Very small size proteoliposomes (VSSP) were obtained from the Center of Molecular Immunology, Havana, Cuba and produced as described by Estevez et al. [12]. The $Al(OH)_3$ adjuvant (Alhydrogel) was obtained from Brenntag Biosector, Denmark.

2.3. Vaccine formulations

We used in all experiments 120 μg of $LALF_{32-51}$-E7 per mouse either alone, mixed with 200 μg of VSSP ($LALF_{32-51}$-E7 + VSSP) or $Al(OH)_3$-adsorbed [$LALF_{32-51}$-E7 + $Al(OH)_3$]. We used 0.6 mg Al^{3+} per dose in all formulations containing $Al(OH)_3$. The adsorption of $LALF_{32-51}$-E7 to $Al(OH)_3$ was estimated to be >95%.

2.4. Mice and immunizations

Female C57BL/6 mice, 6–8 weeks old (CENPALAB, Cuba) were injected subcutaneously in the right flank twice or four times at 7-days intervals. The experimental methods were approved by the Animal Care and Use Committee of the Center for Genetic Engineering and Biotechnology and were conducted in accordance with the Health Guide for the Care and Use of Laboratory Animals (HGCULA).

2.5. Selection of tumourigenic challenge cell line TC-1* and implantation of tumors in mice

The TC-1 cell line was kindly provided by Dr. T.C. Wu (Johns Hopkins University, Baltimore) and maintained as described by Lin et al. [13]. To prepare more tumourigenic TC-1 cells, mice were challenged subcutaneously with TC-1 cells in the right leg and when the tumors reached a size of 1.0–1.5 cm they were excised and further sub-cultured. The released cells were expanded in medium and then subjected to two subsequent passages in mice. The resulting cells were named TC-1*.

For the experiments, mice were challenged subcutaneously in the right leg with 5×10^4/mouse of TC-1* cells. Starting 5–7 days later and every 2 days thereafter the area was observed and palpated for the presence of a tumor nodule. When the presence of tumors was apparent, their diameters were measured using electronic digital calipers (Kell-Strom, Canada). Tumor volumes were calculated according to: (length × $width^2$)/2 [14].

2.6. ELISA assay

A pool of sera in each group of mice was prepared seven days after the last immunization. The high-binding plates (Costar) were coated for 16 h at 4 °C with 5 μg/mL of E7 protein or 10 μg/mL of $LALF_{32-51}$ peptide or were coated for 2 h at 37 °C with 5 μg/mL of $LALF_{32-51}$-E7 protein. Serial dilutions of mouse sera were added in duplicates. The reaction was developed for 10 min with 3,3′,5,5′-Tetramethylbenzidine substrate solution (Invitrogen, USA) and the absorbance read at 450 nm in a microplate reader (Sensident scan, Labsystems, Finland). Sera were designated positive at a given dilution if the absolute optical density was equal to five standard deviations (SD) above the mean optical density of control wells.

2.7. Evaluation of IgG subclasses

The plates were coated with 5 μg/mL of E7 protein. The IgG2c and IgG1 subclasses in sera were determined by ELISA using the antibodies from SouthernBiotech and following the manufacturer's instructions.

2.8. IFN-γ ELISPOT

IFN-γ ELISPOT assay was performed using a Mouse IFN-γ $ELISpot^{PLUS}$ kit (Mabtech, Sweden). Pooled splenocytes from immunized mice were prepared seven days after the last immunization in serum-free culture medium (Cellular Technology Ltd.). Triplicates cultures (2×10^5 splenocytes per well) were incubated in a round-bottom plate with 10 μg/mL of $E7_{49-57}$ peptide, 10 μg/mL of $E7_{48-57}$ peptide or medium alone for 48 h at 37 °C/5% CO_2. Then, all the content of this plate was transferred to the ELISPOT pre-coated plate and incubated for 24 h at 37 °C/5% CO_2. The incubations with the antibodies and the development of the enzymatic reaction were done as described by Granadillo et al. [8]. The spots were counted using AELVIS ELISPOT reader and software.