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Cell mediated immune response elicited in mice after immunization with the P64k meningococcal protein: epitope mapping

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Abstract

The P64k protein of *Neisseria meningitidis* has been reported as an immunological carrier for weak immunogens. This investigation was aimed at characterizing the T-cell response produced in primed mice and at identifying T helper cell epitopes within this molecule. BALB/c mice subcutaneously immunized with the recombinant antigen provided inguinal lymph node cells (LNC) that proliferated in the presence of P64k in a dose-dependent manner. Proliferating cells secreted IL-4 while the concentration of IL-12 remained unaltered in the culture supernatant. By testing a panel of 59 overlapping synthetic peptides spanning the entire sequence of the antigen a T-cell determinant was localized. Prime-boost and lymphoproliferation experiments, conducted with highly purified synthetic peptides, confirmed that the segment including amino acids 470–485 comprises a T-cell epitope within the P64k molecule.
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Keywords: P64k; Meningococci; T-cell response; Helper T-cell epitope

1. Introduction

Mapping of T-cell epitopes and characterization of cellular response are important for vaccine development, understanding of disease pathogenesis, monitoring of disease development and analysis of immune response in general. *Neisseria meningitidis* is a pathogen responsible of a great number of cases of bacterial meningitis worldwide [1]. Our group has previously identified and cloned the *lpdA* gene, which encodes the P64k protein of *N. meningitidis* [2]. The P64k protein has been expressed as a soluble antigen in *Escherichia coli*, accounting for more than 20% of the total cell proteins. Pure recombinant antigen has been extensively characterized [3,4]. The *lpdA* gene, as well as the P64k protein, is highly conserved all through the studied strains of meningococci.

Due to its relatively high molecular weight, demonstrated immunogenicity [5] and availability, P64k was employed as a carrier protein for poorly immunogenic peptides and *N. meningitidis* serogroup C polysaccharide with good results [6]. Moreover, the recombinant P64k acted as a carrier in a novel cancer vaccine, developing a self-reactive antibody response against human Epidermal Growth Factor, in patients with histologically proven malignant carcinomas [7]. This antibody response is desirable during the active immunotherapy with Epidermal Growth Factor in cancer patients.

B-cell epitopes present in P64k, recognized by monoclonal and polyclonal antibodies developed in animals, have been reported [5,8]. Having into account the prospective use of the recombinant P64k protein as a carrier in prophylactic, as well as in therapeutic vac-

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cines, we wanted to further characterize the immune response against this bacterial protein. This investigation was aimed at characterizing the T-cell response produced in primed mice and at identifying T-helper cell epitopes within this molecule.

2. Materials and methods

2.1. Mice

BALB/c (H-2^d) mice were obtained from Centro para la Producción de Animales de Laboratorio (CENPALAB), Havana, Cuba, with the exception of mice employed in the T-cell epitope scanning. Those mice were supplied by the Center for Genetic Engineering and Biotechnology of New Delhi, India. All mice (female) were used at age 6–8 weeks.

2.2. In vitro proliferation assay with P64k

The purified P64k protein employed in this assay passed the pyrogenicity test, performed in accordance with the requirements of the United States Pharmacopoeia [9]. Mice were subcutaneously immunized at the base of tail with 20 μg per mouse of recombinant protein as an emulsion in Complete Freund's Adjuvant. Seven days later, three mice were killed, inguinal lymph nodes were removed, pooled and the cells were dispersed manually. Once washed and counted, viable cells (LNC) were cultured at a density of $3–5 \times 10^5$ cells per well in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 $\mu g\,ml^{-1}$ gentamicin and 50 μM 2-mercaptoethanol, in 96-well Costar plates. Different concentrations of P64k diluted in the same medium were added to triplicate wells to give a total volume of 200 μl per well. Controls were run with complete culture medium or 2.5 $\mu g\,ml^{-1}$ Concanavalin A, to demonstrate the proliferative capacity of the cells. Plates were incubated at 37 °C in 5% CO_2 in air. After three days, cells were pulsed with 0.5 μCi of [^{3}H]thymidine per well for an additional 15 h, harvested with an Skatron Automatic Cell Harvester (Skatron, Norway), and counted in a liquid scintillation counter. Results of proliferative assays were expressed as stimulation index (SI), i.e., the ratio of counts per minute in culture with stimulus to counts per minute in control cultures without stimulus. At least, three parallel experiments were run, with three mice per variant, each. An SI >2 was considered positive.

2.3. Cytokine quantitation

Nine mice were sensitized as described in Section 2.2. Then, LNC from three mice were obtained, pooled and cultured as indicated, in the presence of P64k (20, 5 and 0.5 $\mu g\,ml^{-1}$), complemented RPMI 1640 medium or Concanavalin A. Supernatants of triplicate cell cultures were collected and pooled 24 and 72 h later for Interleukin-4 (IL-4) and IL-12 measurements. IL-4 and IL-12 concentrations were determined by a capture ELISA assay using paired monoclonal antibodies specific for murine IL-4 and IL-12, supplied by R&D Systems, USA. ELISA assays were done following the manufacturer instructions, with recombinant cytokines (provided with the assay) used for standard curves with the respective monoclonal antibodies. The experiment was conducted three times.

2.4. Localization of T-cell epitopes on P64k

Initially, 59 overlapping synthetic peptides that encompassed the full-length 596 amino acids of the protein were synthesized, using the Multipin Cleavable Peptide Kit (Chiron Technologies, Australia). Peptides were synthesized as 20-mers, with adjacent peptides overlapping by 10 amino acids. LNC of P64k-sensitized mice were tested for proliferation in the presence of the peptides, as described in Section 2.2. In separate experiments, all the peptides were tested in three different concentrations (30, 10 and 1 $\mu g\,ml^{-1}$). To perform the mapping with all the peptides simultaneously, 10 $\mu g\,ml^{-1}$ was the peptide concentration selected.

Later, synthetic peptides P1 (20-mer), P48 (25-mer), P48A, P48B and P48C (15-mer, each), derived from the amino acid sequence of P64k, were synthesized using Fmoc chemistry on Rink resin. Crude peptides were purified to at least 95% purity by reverse phase-high performance liquid chromatography. All the proliferative response assays with synthetic peptides were performed as described above, employing 50 μg of peptide or 20 μg of P64k emulsified in Complete Freund's Adjuvant for the sensitization. A non-related peptide (C13), derived from the sequence of the PorB meningococcal protein was synthesized using the same approach. This peptide was employed as a negative control in several experiments.

2.5. Prime boost immunization experiment

The prime-boost immunization in mice was performed by sensitizing seven mice per group with either 50 μg of peptide or 1 μg of recombinant P64k protein in Complete Freund's Adjuvant and boosting the animals, seven days later, with 1 μg of P64k in Incomplete Freund's Adjuvant. Peptide C13 was employed as a non-related control. Serum samples were taken once a week during 35 days preserved at −20 °C, and tested for the presence of anti-P64k antibodies by an standard ELISA test. The plates were coated with P64k (1 $\mu g\,ml^{-1}$) and the immunoassay continued as described elsewhere [10].

2.6. Statistical analysis

A one-way analysis of variance, followed by a Bonferroni's Multiple Comparison test was used to analyze the significance of the results obtained in IL-4 and IL-12 quantitation. Data were determined to be significant at $p < 0.05$.

3. Results

3.1. Cellular immune response against recombinant P64k

BALB/c mice were subcutaneously immunized with P64k (20 μg per animal) in Complete Freund's Adjuvant. Seven days later, inguinal LNC were tested for proliferation in the presence of this recombinant antigen, and a dose-dependent lymphoproliferation was observed (Fig. 1). The assay was performed as well with LNC obtained on day 14, seven days after the second dose of recombinant protein. The same pattern of proliferative response was obtained (data not shown).

In the same experimental model, the presence of IL-4 and IL-12 in cell culture supernatants was studied. In Fig. 2 it can be observed that the level of IL-4 secreted 24 h after the antigenic stimulation was negligible. However, the amount of this cytokine increased, and after 72 h of stimulation with P64k (20 $\mu g\,ml^{-1}$) the amount of IL-4 was statistically different ($p < 0.05$) from the level secreted in the absence of antigen. Nonetheless, the concentration of IL-12 remained unaltered in the culture supernatant all over the duration of the experiment.

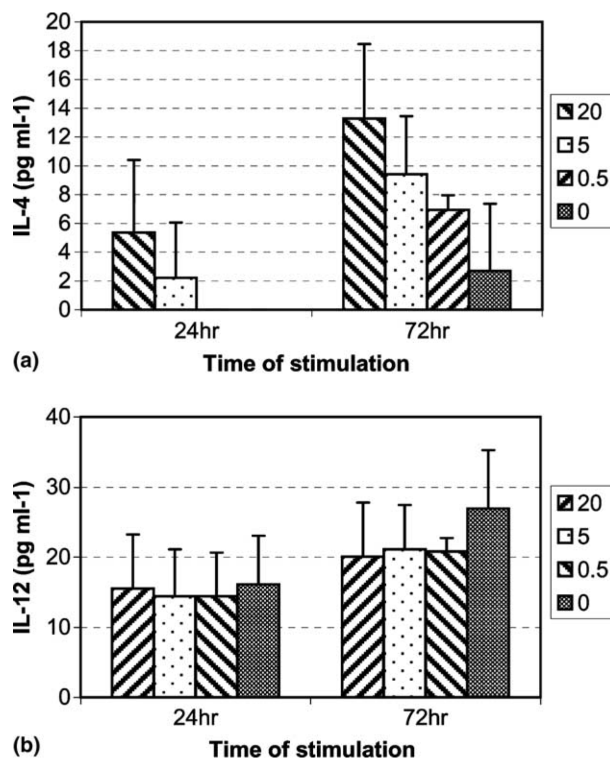

Fig. 2. IL-4 (a) and IL-12 (b) detected in culture supernatants of primed LNC stimulated with P64k. Three mice were sensitized with P64k and LNC were obtained from inguinal lymph nodes. Cells were stimulated with different concentrations of the same antigen. At the times indicated in the x axis the supernatants were collected. The concentrations of IL-4 and IL-12 were determined by ELISA (R&D Systems). They represent the average of three independent experiments. Numbers in the legend indicate the concentrations of P64k ($\mu g\,ml^{-1}$) used to stimulate LNC.

3.2. Mapping of T-cell epitopes in P64k

Proliferation of P64k-primed LNC was also observed when the cells were stimulated with two overlapping peptides (peptides 48 and 49) from a library of 59 peptides (20-mers each) spanning the entire sequence of P64k protein. Fig. 3 shows the results achieved in such experiments, when the cells were simultaneously stimulated with 10 $\mu g\,ml^{-1}$ of each peptide.

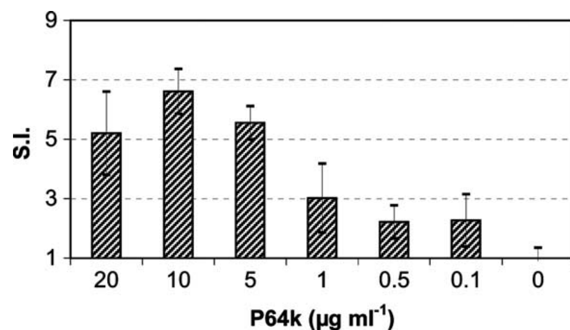

Fig. 1. P64k-stimulated dose-dependent proliferation (±SD) of murine LNC. BALB/c mice were sensitized with a single injection of P64k in Complete Freund's Adjuvant. LNC were obtained from inguinal lymph nodes of three mice immunized with the recombinant protein. The results shown are representative of three independent experiments. SI: Stimulation Index.

Starting from this result, two peptides (P1 and P48) were synthesized and purified to continue with the cellular response experiments. P1 corresponds to the N-terminal peptide of the library and P48 is five amino acids longer than the respective peptide 48. Fig. 4 shows the proliferative response given by LNC when P1 and P48 were used to sensitize mice. Only LNC obtained from P48 mice produced a positive proliferative response when challenged with the homologous peptide. The results presented are representative of three independent proliferation assays. When these experiments were carried out in parallel in C57BL/6 mice, neither P1 nor P48 stimulated lymphocyte proliferation (data not shown).

To confirm the results obtained in vitro, the capacity of the peptides P1 and P48 of sensitizing mice for an in vivo secondary response was studied. Fig. 5 shows the

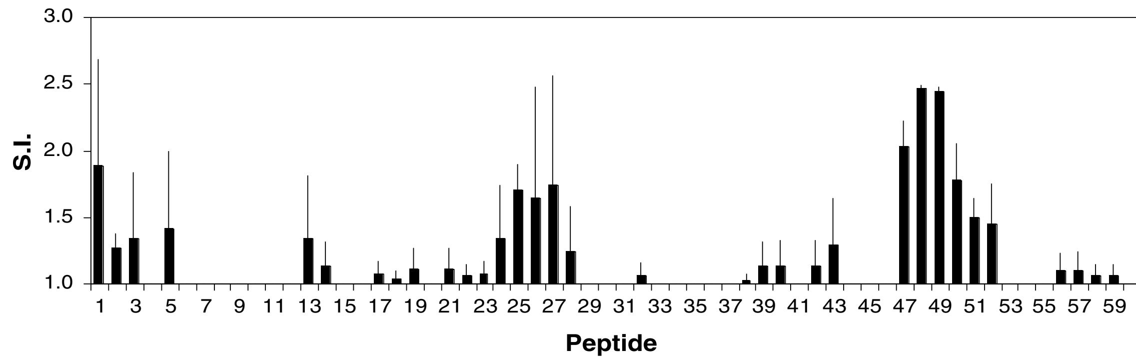

Fig. 3. Lymphocyte proliferation of P64k-sensitized LNC induced by overlapping peptides. LNC were obtained from inguinal lymph nodes of three mice immunized with the recombinant protein. Fifty nine peptides spanning the whole sequence of the meningococcal P64k protein were synthesized and used to stimulate LNC. The peptides were tested at 10 $\mu g\,ml^{-1}$. The results shown are the average (plus standard deviation) of two independent experiments. SI: Stimulation Index.

P64k-specific antibody response elicited in mice primed with either P1 or P48 and boosted, seven days later, with 1 μg of recombinant antigen. P48 sensitized the animals for an anti-P64k secondary response, while P1 behaved like the non-related peptide.

Finally, three overlapping peptides (P48A, P48B, and P48C), spanning the P48 sequence, were tested for proliferation in homologous peptide-sensitized mice (Fig. 6). To determine the minimal epitope, these peptides were synthesized as 15-mers, with adjacent peptides overlapping by 10 amino acids. The highest proliferative response was obtained against peptide P48A, which includes amino acids 470–485. When primed LNC were challenged in vitro with peptide P48 similar results were observed (data not shown).

4. Discussion

The humoral immune response is an essential defense line against the microorganisms. However, T-cell mediated immunity also plays an important role in protection against pathogens [11]. The CD4+ helper T-cells offer help to B-cells in the production of antibodies and they secrete a series of cytokines that are involved in a variety of immunoregulatory functions or they have a direct effect on the invader microorganism [12].

The inguinal LNC of P64k-sensitized mice showed positive lymphoproliferative response, when challenged during 96 h with the same antigen. This result was expected, because it is accepted that CD4+ T-cells are easily generated after the immunization with soluble

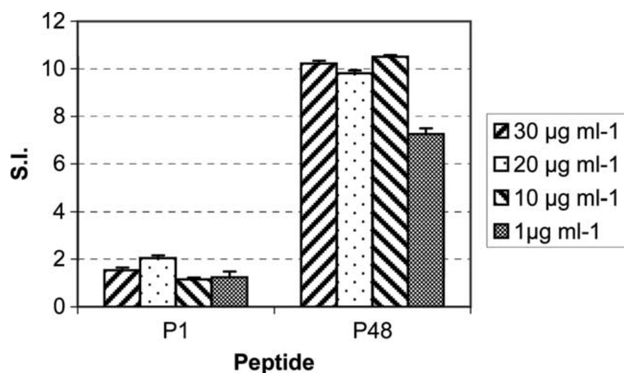

Fig. 4. Lymphocyte proliferative response of peptide-primed LNC. BALB/c mice were immunized with 50 μg of peptide P1 or P48 emulsified in Complete Freund's Adjuvant. Seven days later, LNC were stimulated with several concentrations of the homologous peptide. The results shown are representative of three independent experiments. SI: Stimulation Index.

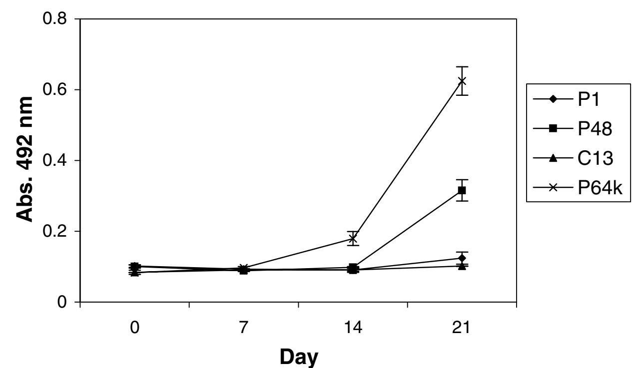

Fig. 5. A single dose of peptide P48 primed for a P64k-specific secondary antibody response. Mice ($n = 7$) were subcutaneously immunized with 50 μg of peptide (1 μg of P64k in the control group) in Complete Freund's Adjuvant. Seven days later, animals were boosted with 1 μg of P64k in Incomplete Freund's Adjuvant. P64k-specific antibodies were determined by ELISA in serum samples obtained at times indicated in the x axis. In the legend are shown the immunogens used for priming. The results shown are the average (plus standard deviation) of individual animals in each group.

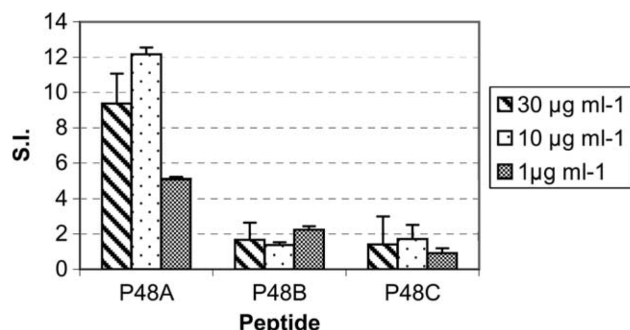

Fig. 6. P48A produced a proliferative response as strong as the parent peptide P48. Three overlapping peptides spanning the sequence of peptide P48 were synthesized. Mice were sensitized with 50 μg of peptide emulsified in Complete Freund's Adjuvant. Seven days later, LNC were obtained from three mice and were stimulated with homologous peptide. The results shown are representative of three independent experiments. SI: Stimulation Index.

antigens [13], which is the case of recombinant P64k protein.

It is well-known that there are at least two subpopulations of CD4+ T-cells, based on its functions and on cytokine secretion [14]. In this study, we found that murine P64k-primed LNC, stimulated in culture with the same antigen (20 $\mu g\,ml^{-1}$), secreted levels of IL-4 higher than those secreted by unstimulated cells, while the amount of secreted IL-12 did not differ from the control. It should be taken into account that mice were primed with the antigen in Complete Freund's Adjuvant, which is one of the most potent adjuvants described so far [15]. In general, adjuvants modulate the immune responses by prolonging the antigen presentation, increasing the expression of MHC I and II, up-regulating adhesion molecules and augmenting cytokine production [16]. Adjuvants can also modulate the immune response to different T-helper cells [17]. In this study, the use of Freund's Adjuvant might have influenced in the amount of IL-4 that was detected. Use of another adjuvant may result in a completely different pattern of secreted cytokines and a dissimilar Th1/Th2 ratio. Moreover, it has been suggested that Freund's Adjuvant might partly unfold the P64k protein [18], which could facilitate antigen processing. It appears that a primary goal of antigen processing is to unfold the protein to expose residues that are buried in the native conformation. Berzofsky and co-workers have found that, although the antigen presenting cell usually accomplishes this task by proteolytic cleavage of the protein, artificial unfolding without proteolysis is sufficient [19].

The production of cytokines exclusive, or almost exclusive, of a particular population of T-cells is a reliable method for the demonstration of the induction in vivo of such a population of cells [20]. The secretion of IL-4 in this type of assay is considered as an indicative of a Th2 type of response [21]. The Th2 cells are considered the true helper T-cells; the cytokines secreted by them play a key role in the switch of class of immunoglobulin and in the differentiation of B-cells, in particular for the production of IgE, IgA and IgG1 antibodies [22]. Due to the heterogeneity of the cells in in vitro culture, alternative methods of monitoring cytokine production, like flow cytometric analysis of intracellular staining, could be performed to confirm the type of response (Th1 or Th2) against P64k elicited in mice.

Identification of antigenic determinants within protein molecules is of theoretical importance in understanding the fundamental interactions involved in immune responses and of potential practical value in the design of subunit vaccines and diagnostic reagents [23]. In 1990, the pin synthesis technique of synthesizing and screening large number of peptides was extended to the analysis of T-cell determinants [24].

The T-cell response to many whole proteins is focused on a limited number of possible determinants, which can be termed immunodominant. In addition, other "less dominant" determinants, the subdominant or cryptic epitopes, can be found within it [25]. To identify T-cell epitopes on P64k, we obtained overlapping peptides that encompass the full length of the protein and test their reactivities. LNC of mice primed with the recombinant P64k protein proliferated after stimulation with two overlapping peptides (peptides 48 and P49), indicating that the minimal T-cell epitope may be contained in the shared amino acid segment. Using this method, we found only one T-cell determinant. This is in agreement with results published by others, since there are parasitic [26], viral [27] and even meningococcal proteins [28] that possess one or two immunodominant T-cell epitopes, detected when this kind of determinants was mapped in mice.

The data, obtained when the highly purified P48 peptide was employed to immunize mice and to stimulate primed LNC, validated the results achieved with the Multipin system. The N-terminal segment of P64k (which includes the P1 peptide) has been frequently employed, fused to other proteins, to increase the expression of heterologous antigens in *E. coli*, resulting in hybrid molecules [29]. To be included in vaccines, it was desirable that a T-cell epitope were present in that region. However, instead of P1, it was P48 which gave evidences of containing such an epitope in several independent experiments. Moreover, P48 contains a T-cell determinant in humans, according to some T-cell epitope prediction methods implemented as World Wide Web servers. For example, as predicted by using the softwares ProPred [30], MHC-Thread [31] and SYFPEITHI [32], P48 encloses binding motifs for HLADRB1*0401, HLA DR B1*0426, HLA DR B1*0101, HLA DR B1*0701 and other alleles.

The level of response against this peptide seems to be genetically determined, since it induced a strong lymphocyte proliferative response in primed cells of BALB/c mice (H-2^d), but there was no response in primed cells of C57BL/6 mice (H-2^b), cultured and stimulated in parallel. This is in agreement with a prediction algorithm for I-A^d binding motifs [33] which predicts one motif within P48 (PGVAYTSPE) and no binding motif for I-A^b within it. According to this prediction the amino acids P, A, T and E could bind in the pockets of I-A^d and failed to bind in the pockets of I-A^b. The immunogenicity of a given epitope is dependent upon three factors: the generation of the appropriate fragment, the presence of an MHC molecule that binds this fragment and the presence of T-cells capable of recognizing the complex [34]. Most experiments with mice indicate the lack of an appropriate MHC molecule as the most frequent cause of unresponsiveness. Indeed, MHC molecules are highly polymorphic and it has been shown that a given peptide can bind to only one or few alleles [35–37].

To further restrict the amino acid segment able of inducing proliferation in murine LNC, we tested three overlapping peptides, spanning the sequence of P48. We found that peptide P48A, encompassing residues 470–485 of the entire protein, produced a proliferative response as strong as the parent peptide, indicating that this 15 amino acid segment (IPGVAYTSPEVAWVG) contains a T-cell epitope for BALB/c mice. It is the first time that the presence of such an epitope is demonstrated for this meningococcal protein. In addition, when we looked at the reported B-cell epitopes, determined for this antigen [5], we observed that this region comprises also a B-cell determinant.

Based on the results of this study, and on elements well-established in the literature, we could presume that meningococcal P64k can be classified as a T-cell dependent antigen, able of stimulating the Th2 subpopulation of immune cells when Complete Freund's Adjuvant is employed for the immunization of mice. The knowledge existing on this protein, at the molecular level, as well as its safety and immunogenicity demonstrated in healthy volunteers [38] supports its use not only in conjugate vaccines, but also in hybrid molecules, resulting from the genetic manipulation of this antigen in order to insert in it protective epitopes from foreign antigens.

Acknowledgements

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