

Effect of two synthetic peptides mimicking conserved regions of equine infectious anemia virus proteins gp90 and gp45 upon cytokine mRNA expression

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Abstract Gp90 and gp45 synthetic peptides, which mimic conserved sequences of native viral proteins, are recognized by antibodies to equine infectious anemia virus (EIAV) in asymptomatic carrier horses and generate humoral and cellular responses in immunized mice. Cytokine mRNA levels were evaluated in equine peripheral blood mononuclear cells (PBMCs) after in vitro stimulation with gp90 and gp45 with the aim of determining the cytokine profile associated with the proliferative response. Stimulation index (SI) values indicate that 100 and 60% of EIAV-infected horses recognized gp90 and gp45, respectively. A strong positive correlation was found between IL-12p40 and SI, IFN- γ and SI, and IL-12p40 and IFN- γ ($p < 0.001$). These results suggest the presence of specific memory cells that would contribute to maintain reinfection resistance and that conserved viral regions represented by gp90 and gp45 synthetic peptides may be good candidates for inclusion in vaccine strategies against EIAV.

Introduction

Equine infectious anemia virus (EIAV), a member of the family *Retroviridae*, establishes a persistent infection in the Equidae family that is distributed worldwide. The disease is characterized by recurrent febrile episodes associated with viremia, anemia and thrombocytopenia [1, 2]. After resolution of the primary viremia, most animals develop chronic EIA; then the disease progresses to an asymptomatic stage in which the animals remain free of clinical symptoms but become life-long EIAV carriers [3–5]. A strict control of replication mediated by the immune system has been demonstrated at this stage, and long-term inapparent carrier horses are highly resistant to further exposure to EIAV [6, 7].

EIAV contains two envelope glycoproteins, gp90 and gp45, which together with the major core protein p26, are the primary immunogens during persistent infection [8, 9]. In previous work, we demonstrated that gp90 and gp45 synthetic peptides, which mimic conserved sequences of native viral proteins, are specifically recognized by antibodies and lymphocytes from naturally-infected EIAV-inapparent carrier horses and that gp90 and gp45 are able to generate humoral and cellular responses in immunized mice [10, 11]. The gp90 synthetic peptide represents a highly conserved region located close to the C-terminal domain of gp90; the gp45 synthetic peptide overlaps the immunodominant epitope CIERTHVFC, between the cysteine residues 536 and 544. This potential loop constitutes the principal immunodominant domain (PID) in the lentiviruses [12].

Cytokines play a key role in immunity against pathogens by controlling the type of response and therefore its effector mechanisms. Type I interferons induce resistance to viral replication and promote the expression of MHC

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class I molecules, whereas IL-12, together with IFN- γ and IL-4, are the major regulators of Th1 versus Th2 responses [13–16]. The prevalence of Th1 responses against EIAV in acute and chronic infections has been documented [5]. Studying inhibitory cytokines, such as TGF- β , is also important because their immunoregulatory functions are essential for all immune responses.

Based on previous information and on the relevance that cytokines would have after T-cell proliferation, the aim of this work was to evaluate the profile of cytokines associated with proliferative response in peripheral blood mononuclear cells (PBMCs) after *in vitro* stimulation with gp90 or gp45 synthetic peptides. For this purpose, we determined the levels of IL-12p40, IFN- α , IFN- γ , IL-4, and TGF- β by semiquantitative RT-PCR after stimulation with gp45 and gp90 in PBMCs isolated from naturally-infected asymptomatic carrier horses. We also analyzed constitutive levels of mRNA coding these cytokines in equine PBMCs.

Materials and methods

Peptide synthesis and characterization

The synthesized peptides, identified as gp90, ET-WKLVKTSQVTPLPISSEANTGLIRHKR (aa 409–436), and gp45, ERQQVEETFNLIGCIERTHVFCFTG (aa 523–547), represent two highly conserved and immunodominant regions of EIAV. Both peptides were synthesized manually on solid phase using 9-fluorenylmethoxycarbonyl (Fmoc) chemical strategies and following standard protocols [17, 18] by the Departamento de Química Orgánica of the Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral. The gp45 peptide, used in the cyclic form, was prepared by oxidation of the Cys residues with I₂ solution, using peptide concentrations of 0.1 mg/ml in 0.03 M ammonium bicarbonate buffer, pH 7.9, to minimize unwanted dimerization and oligomerization. The reactions were monitored by the Ellman colorimetric assay and, at the same time, by high-performance liquid chromatography (HPLC) [19].

Horses

EIAV-infected carrier horses [EIAV(+) group]: Twenty-two adult mixed-breed horses, naturally infected with uncharacterized EIAV strains, at the asymptomatic stage of infection, were included in this study. All horses had antibodies to EIAV as determined by agar gel immunodiffusion (AGID) and ELISA tests [10, 20], evaluated once a year for at least 5 years.

Control horses [EIAV(–) group]: Seventeen adult mixed-breed horses that were negative in AGID and

ELISA tests were employed as uninfected controls. These animals came from a racehorse-breeding ranch with 10-year absence of clinical and serological disease, checked twice a year.

Blood samples

Equine peripheral blood samples were collected aseptically by jugular venipuncture and put in sterile tubes. PBMCs were obtained by centrifugation through Histopaque-1077 (Sigma, St. Louis, MO). Cells were washed twice with PBS, counted and conserved at –80°C for RNA extraction, or resuspended for culture assays.

Total RNA isolation

PBMC pellets were homogenized in TRIzol reagent (Gibco, Carlsbad, CA), and total RNA was isolated according to the manufacturer's protocol and dissolved in RNase-free water. RNA was quantified by using a spectrophotometer at 260 nm, and purity was assessed by determining the OD ratio 260/280. The integrity of the 18S and 28S rRNA was determined visually by denaturing agarose gel electrophoresis.

Reverse transcription of equine mRNA into cDNA

For each sample, 2 μ g of total RNA was used to synthesize single-stranded complementary DNA with MMLV reverse transcriptase (200 U; Promega, Madison, WI) and random primers (1 μ g; Invitrogen, Brazil). The reaction was carried out in a volume of 25 μ l in the presence of Recombinant Rnasin Ribonuclease Inhibitor (25 U; Promega, Madison, WI), 1x buffer (50 mM Tris HCl, pH 8.3; 3 mM MgCl₂; 75 mM KCl; and 10 mM dichlorodiphenyl trichloroethane) and a mixture of deoxyribonucleotide triphosphates (dNTP, Invitrogen, Brazil) at a final concentration of 2 mM at 37°C for 1 h. The cDNA samples were kept at –20°C until they were used.

PCR amplification

The methodology used for semiquantitative PCR reactions was based on reports by Van den Hoven et al. and Fraser et al. [21, 22]. The constitutively expressed housekeeping gene β -actin was used as an internal control and as a reference for normalizing the expression of target mRNAs [5, 22–25]. The oligonucleotide primers used for the detection of cDNA specific for equine IL-12p40, IFN- α , IFN- γ , IL-4, TGF- β and β -actin were designed from the published nucleic acid sequences available from GenBank/EMBL databases, with DNASTar software version 5.0. To exclude product amplification

from genomic DNA, primers were designed to anneal at separate exons.

PCR reactions were performed in 25 μ l total volume containing: 1x PCR buffer (20 mM TrisHCl, pH 8.4; 50 mM KCl), 2 mM MgCl₂, 0.2 mM dNTP mix and 1.25 U of Taq DNA polymerase (Invitrogen, Brazil), 10 pmol of each primer and 2 μ l of reverse-transcribed samples containing template cDNA.

Kinetic studies were conducted to verify that amplification of all products was in log phase (data not shown). Samples were amplified as follows: an initial denaturation step at 95°C (120 s), *n* cycles consisting in denaturation at 95°C (60 s), annealing (60 s) and polymerization at 72°C (90 s) with a final extension at 72°C (10 min). Primers, annealing temperatures, number of PCR cycles and expected PCR fragment sizes are described in Table 1.

After PCR, fragments were separated by agarose gel electrophoresis (2% agarose in 0.5x Tris-acetate-EDTA) in the presence of ethidium bromide to facilitate visualization of fragments. A 100-bp DNA ladder standard (PBL, Quilmes, Argentina) was used as a size marker. PCR products were sequenced to confirm the specificity of the oligonucleotide primers. The intensity of bands was analyzed in arbitrary units with the public domain Scion Image program (http://www.scioncorp.com/pages/scion_image_windows.htm). Cytokine signal intensity ratios were calculated as follows: fluorescence of cytokine/ fluorescence of β -actin = corrected fluorescence ratio, calculated for each amplification reaction. In addition, in the case of stimulation assays, corrected fluorescence ratio of peptide-stimulated PBMCs/corrected fluorescence ratio of non-stimulated PBMCs = normalized fluorescence ratio was calculated for each target cytokine.

Stimulation assays

Proliferation assays were performed in PBMCs obtained from blood of EIAV-infected or uninfected horses in the presence of gp90 or gp45 synthetic peptides. After stimulation, cytokine mRNA production was measured.

For mRNA measurement, cultures were performed with 4×10^6 cells/well in 24-well plates and incubated for 4 h at 37°C in 5% CO₂ with phytohemagglutinin (PHA) (10 μ l, Gibco), purified gp90 peptide or purified gp45 peptide (5 μ g/ml) in a 2-ml final volume. After culture, cells were centrifuged at 1,800 rpm and cell pellets were conserved at -80°C for RNA isolation. As cytokine production depends on time elapsed from the beginning of stimulation, to determine the kinetics of each cytokine mRNA production, cultures were previously performed for 4, 24, and 48 h. Cytokine mRNA levels were at a maximum at 4 h of culture in all cases, except for TGF- β and IL-4, which remained constant at all time points evaluated. mRNA semi-quantification was performed as described previously.

Lymphoproliferation assays were performed as described by Soutullo et al. [11]. Briefly, PBMCs (2×10^5) were added to each well of 96-well round-bottomed microplates and incubated for 6 days at 37°C in 5% CO₂ with phytohemagglutinin (PHA) (0.5 μ l, Gibco), purified gp90 peptide or purified gp45 peptide (5 μ g/ml), in 100 μ l of complete RPMI medium supplemented with 50 UI/ml penicillin, 50 μ g/ml streptomycin, 2 mM glutamine, and 10% fetal bovine serum. Cell proliferation was determined by a colorimetric immunoassay, based on the measurement of bromodeoxyuridine (BrdU) uptake during DNA synthesis. The BrdU ELISA was performed following the manufacturer's instructions (cell proliferation ELISA

Table 1 Primer pairs, gene sequence accession numbers, PCR conditions and product lengths for the detection of IFN- α , IL-12p40, IFN- γ , IL-4, TGF- β , and β -actin mRNA

Primer name	Primer sequence (5'-3')	Accession number	Annealing <i>T</i> (°C)	Cycles	Product size
IFN- α upper	GGCAACACAAGGGTCTTGAT	A33683, A33687	55	35	275
IFN- α lower	CTCAGACAGGCTTCCAGCTC	A33697, A33699 ^a			
IL-12 p40 upper	CACAAAGGAGGCGAGGTTCTGAGC	Y11129	64	37	449
IL-12 p40 lower	TTGGGTGGGTCTGGTTTGATGATG				
IFN- γ upper	GTGTGCGATTTTGGGTTCTTCTA	U04050	54	32	431
IFN- γ lower	GACTCCTCTTCCGCTTCCTCAG				
IL-4 upper	GATTCAGCTCTGGTCTGCT	AF305617	55	37	311
IL-4 lower	CAGTACAGCAGGTCCCGTTT				
TGF- β upper	AGGCTCAAGTTAAGCGTGGA	X99438	55	35	409
TGF- β lower	CGCAGCAGTCTTCTCTGTG				
β -actin upper	ATGATATCGCCGCGCTCGTGGTC	AF035774	60	23	343
β -actin lower	TTGGGTGGGTCTGGTTTGATGATG				

^a Primer pair for IFN- α was designed to detect the four equine isoforms of this cytokine

SYSTEM. Pharmacia Biotrak, Piscataway, NJ). Briefly, cells were pulsed with 10 μl /well of 100 μM BrdU solution during the fourth day after the initial stimulation. Twenty-four hours later, plates were centrifuged and cells were denatured and incubated for 120 min with mouse anti-BrdU mAb conjugated to peroxidase (1:100). Conjugated antibody was removed and a substrate solution was added. After 30 min, the reaction was stopped by adding 1 M H_2SO_4 solution. The blank corresponded to 100 μl of culture medium with or without BrdU. Absorbance was measured at 450 nm using an automatic ELISA reader (Multiskan EX). Cell proliferation was calculated as $\text{SI} = (S - C)/C$; where SI is the Stimulation Index and S and C represent the absorbance values for the stimulated and non-stimulated cells (control), respectively.

Statistical analysis

Normality of data was assessed with the Kolmogorov–Smirnov Test. The Pearson test was used to evaluate association between two variables. To assess whether a correlation between two variables was mediated by a third one, the partial correlation test, which eliminates the influence of the third variable, was applied [26]. Data from the constitutive cytokine results from the two animal groups were compared using the Mann–Whitney U test for independent samples. The results were expressed as mean \pm SEM; the significance level was determined at $p < 0.05$. Bar and dispersion graphs are shown as descriptive supplements of methodological results. Statistical analyses were performed with the SPSS version 11.5 for Windows and “R” software of free distribution.

Results

EIAV infection does not modify constitutive mRNA expression of IL-12p40, IFN- γ , IL-4, and TGF- β in PBMCs from asymptomatic carrier horses

With the aim of analyzing the constitutive levels of cytokines and ruling out a possible modification of cytokine expression in PBMCs by EIAV infection, we analyzed constitutive cytokine mRNA levels in PBMCs from EIAV-infected ($n_{\text{EIAV}(+)} = 22$) and healthy horses ($n_{\text{EIAV}(-)} = 17$). Semiquantitative reverse transcription-PCR was applied, and results were expressed as cytokine corrected fluorescence ratios. The analysis of IL-12p40, IFN- γ , IL-4, or TGF- β shows no differences between infected and healthy horses ($p > 0.05$). Noticeably, EIAV-infected horses showed decreased IFN- α mRNA levels when compared with healthy ones ($p < 0.05$) (Fig. 1).

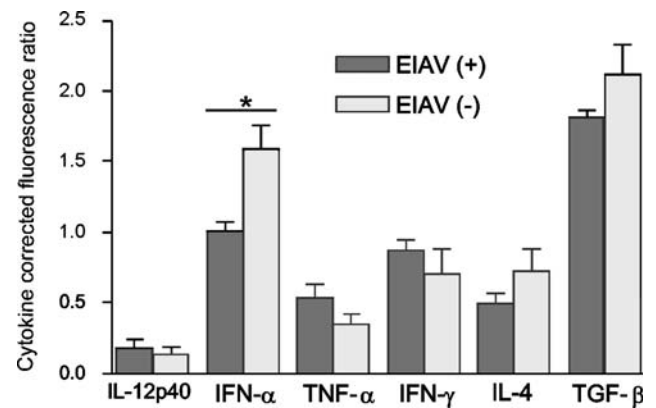


Fig. 1 Constitutive cytokine mRNA expression in PBMCs, represented as cytokine corrected fluorescence ratios, from infected (EIAV(+), $n_{\text{EIAV}(+)} = 22$) and healthy horses (EIAV(-), $n_{\text{EIAV}(-)} = 17$). Bars represent means and error bars represent S.E.M. Asterisks shows statistical differences between infected and healthy groups

Synthetic peptides that mimic viral glycoproteins are able to stimulate proliferation of PBMCs from EIAV-infected horses

With the aim of evaluating the association between cytokine production and the specific proliferative response, we determined SI from PBMC after gp90 and gp45 stimulation. We analyzed PBMCs from EIAV naturally-infected ($n_{\text{EIAV}(+)} = 5$) and healthy horses ($n_{\text{EIAV}(-)} = 5$). Horses were selected randomly and according to availability of samples, after excluded those animals with any constitutive cytokine values outside the ± 2 SD range of the mean value. SI levels, evaluated by the BrdU incorporation method, indicate that all EIAV-infected horses recognized gp90 with SI values higher than 2, and that three of them also recognized gp45. No proliferative response was observed in healthy horses (Fig. 2). In addition, the immune competence of the horses was confirmed with a PHA-stimulation assay.

Specific cellular proliferation is associated with Th1 cytokine production

We determined IL-12p40, IFN- α , IFN- γ , IL-4, and TGF- β mRNA levels in PBMCs from asymptomatic EIAV-infected ($n_{\text{EIAV}(+)} = 5$) and healthy horses ($n_{\text{EIAV}(-)} = 5$) by semiquantitative RT-PCR after specific stimulation. Amplified fragments are shown in Fig. 3a. We analyzed bivariate correlation between SI and cytokine levels and among cytokine levels using the Pearson correlation test, including the total n of 10 animals.

Results showed that SI was highly associated with IL-12p40 and IFN- γ mRNA levels after stimulation with gp90 ($r = 0.894$ and $r = 0.914$, for IL-12p40 and IFN- γ ,

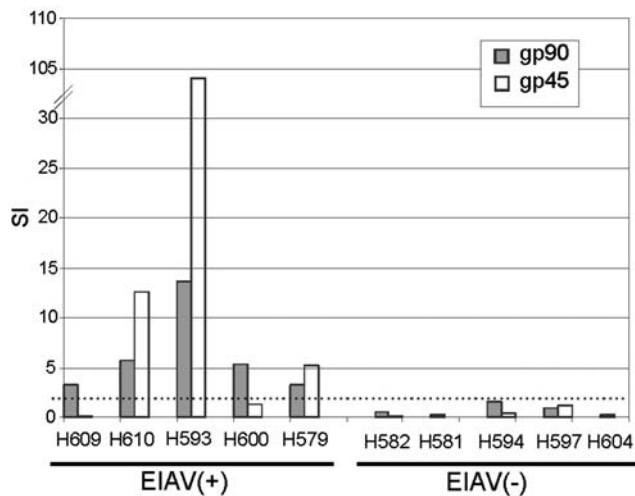


Fig. 2 Determination of BrdU uptake by PBMCs from EIAV-infected ($n_{EIAV(+)} = 5$) and healthy horses ($n_{EIAV(-)} = 5$) stimulated with gp90 or gp45 peptides. The dotted line represents the cut-off value $SI = 2$. No EIAV-specific lymphoproliferation was detected in PBMCs from healthy horses

respectively) and gp45 ($r = 0.991$ and $r = 0.984$, for IL-12p40 and IFN- γ , respectively) ($p < 0.001$) (Fig. 3b). *R* values of the remaining cytokines were low, the highest one being 0.592; this shows a lack of correlation among IFN- α , IL-4 and TGF- β , and between IFN- α , IL-4, TGF- β and SI, IL-12p40 or IFN- γ . Since we found a strong correlation between IL-12p40 and IFN- γ after stimulation with both peptides ($r = 0.982$, $p < 0.001$), we investigated the influence of each cytokine on the association of the other cytokine with SI. The analysis of gp90 stimulation shows that when the variable IFN- γ is controlled, the association between IL-12p40 and SI decreases to non-significant levels ($r = -0.042$, $p = 0.909$). The same occurs with the correlation between IFN- γ and SI, which becomes non-significant ($r = 0.419$, $p = 0.227$) when controlling for IL-12p40 effects. The correlation between SI and IL-12p40 after gp45 stimulation is so strong that it persists even after controlling for IFN- γ influence ($r = 0.735$, $p < 0.05$).

Discussion

In the present work, we observed that two synthetic peptides that mimic conserved regions of EIAV glycoproteins were able to induce a lymphoproliferation strongly correlated with increased levels of Th1 cytokines. In fact, the proliferative response observed in samples from infected animals was associated with the synthesis of the IL-12p40 subunit and IFN- γ mRNA after in vitro stimulation of PBMCs with both gp90 and gp45 synthetic peptides.

First, we analyzed PBMCs from infected and healthy horses to determine if constitutive expression levels of

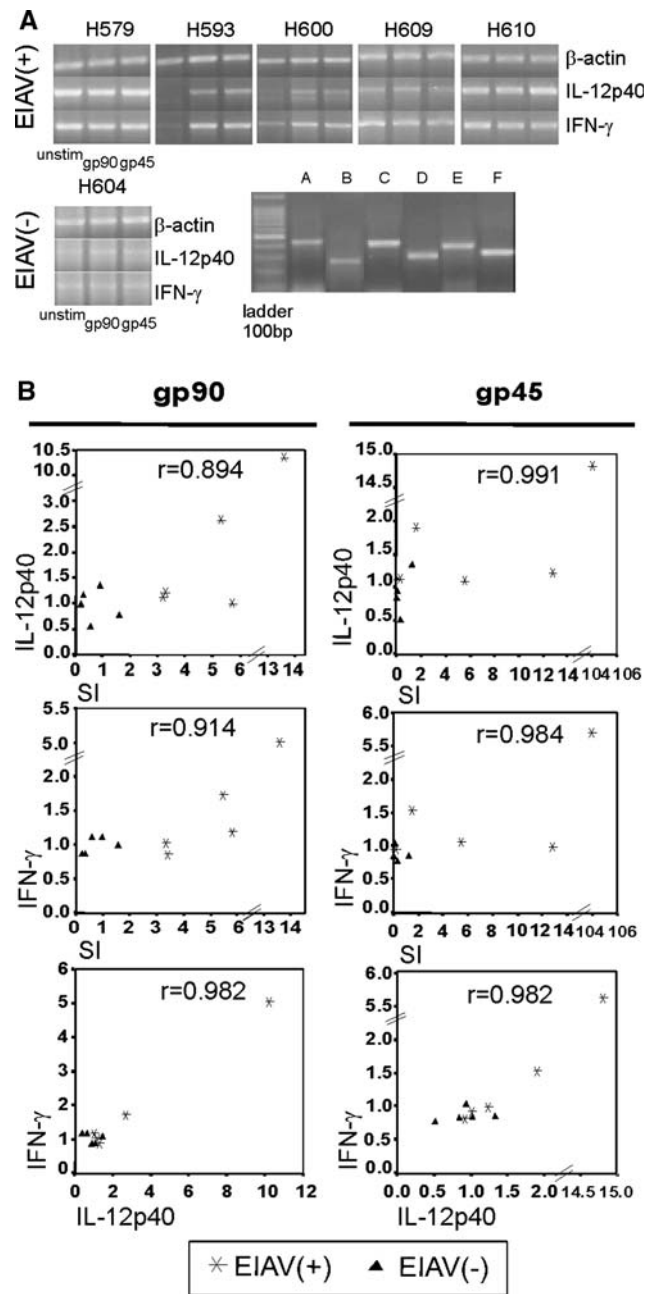


Fig. 3 Semiquantitative RT-PCR analysis of IL-12p40 and IFN- γ mRNA in non-simulated and gp90- or gp45-stimulated equine PBMCs. PCR products from the five naturally-infected carrier horses (EIAV(+)) and from one horse representing the five healthy horses analyzed (EIAV(-)). A, B, C, D, E and F represent the size of IL-12p40, IFN- α , IFN- γ , IL-4, TGF- β and β -actin products, respectively (a). Dot graphs representing bivariate correlations between SI and IL-12p40, SI and IFN- γ , and IL-12p40 and IFN- γ after PBMCs stimulation with gp90 or gp45 synthetic peptides. EIAV-infected (asterisks $n_{EIAV(+)} = 5$) and healthy horses (filled triangle $n_{EIAV(-)} = 5$). All correlation coefficients (*r*) are statistically significant ($p < 0.001$). Cytokine units are expressed as cytokine corrected fluorescence ratios (b)

cytokines are modified by EIAV infection in inapparent carrier horses. No differences in levels of IL-12, IFN- γ , IL-4 and TGF- β were found between the horse groups.

Surprisingly, IFN- α levels were lower in infected horses. Given the scarce information on cytokine expression at this stage of the disease, further studies to determine the mechanisms involved in the decrease of this constitutive cytokine expression are needed.

Most of the reports on EIAV involve experimental infection protocols. To our knowledge, this is the first work that analyses the specific cellular response and the associated cytokine profile in horses naturally and persistently infected with wild viral strains. In agreement with previous work from our laboratory, in which 79 and 47% of asymptomatic carrier horses recognized gp90 and gp45 peptides, respectively (unpublished data), our results suggest that these peptides represent highly antigenic regions that are conserved in most wild strains infecting horses in endemic regions. Moreover, lymphoproliferation in response to gp45 peptide showed higher SI values, which may suggest that this viral region is more immunogenic than that represented by the gp90 peptide. Tagmyer et al. [27] performed a detailed mapping of Env-specific Th and CTL responses from vaccinated horses. They reported several regions that are able to induce proliferation, which span the region containing the gp45 and gp90 peptide sequences. Although we did not aim at assessing response over time, we found that the synthetic peptides assayed were recognized by horses infected for more than 5 years. Thus, our data support the concept of response maturation towards conserved epitopes described by Hammond et al. and Tagmyer et al. [27, 28].

In this work, we observed that specific gp90- and gp45-peptide recognition by PBMCs from infected horses was highly correlated with synthesis of two Th1 cytokines, IL-12 and IFN- γ , both of which are essential in antiviral responses. Similarly, Fraser et al. found that three broadly recognized peptides mimicking viral proteins were able to promote IFN- γ expression by responding CD4+ T lymphocytes from experimentally infected horses. Although they found similar results, their peptides were located in the Gagp26 and Pol viral proteins [5].

The number of samples analyzed in stimulation assays was not enough to make correlation tests in separate groups of horses. However, we could determine such correlation with the different SI values detected in healthy and EIAV-infected horses, which showed that the production of these cytokines occurs as a consequence of a specific response to the peptides. The role of IL-12 and IFN- γ in protection against infections has been reported [23, 29]. Zhang et al. [30] found that high levels of IL-12p40 and IFN- γ mRNA induced by an attenuated vaccine would contribute to a protective immune response against EIAV. Our results suggest the presence of immune memory cells associated with the production of IL-12 and IFN- γ in response to specific stimulation. Epitope-based vaccines are able to

include more than one epitope, and they can also be improved in other aspects relative to design, such as the use of recombinant DNA techniques and the insertion of several immunostimulatory molecules. As a consequence, the identification of epitopes represents an essential step in vaccine design. Taking into account the advantages of epitope-based vaccines, the regions represented by gp90 and gp45 synthetic peptides might therefore be considered for inclusion in vaccine strategies against EIAV. Nevertheless, more studies are needed to evaluate if the sequences studied in this work might be associated with virus control.

Several studies have reported the balance between Th1 and Th2 cytokines in response to different viral infections. Indeed, in the lentiviral infection with HIV in humans, Imami et al. [31] reported that a balanced Th1/Th2 cytokine profile against peptides spanning the p24-gag protein correlates with successful long-term control of infection. However, vaccine designs are aimed at inducing Th1 responses with coordinated CTL and helper T-lymphocyte effector mechanisms and a prevalence of a Th1 cytokine profile. The importance of this work lies in the stimulation of two cytokines, probably involved in protective mechanisms against the virus, by the two peptides analyzed, which represent conserved regions of the two envelope glycoproteins. Since cytotoxic T-cells are a common source of IFN- γ and have been reported to control viremia in acute EIAV infection [32], future research will focus on the study of the cell type that produces this cytokine after peptide stimulation.

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