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Antibodies and PMBC from EIAV infected carrier horses recognize gp45 and p26 synthetic peptides

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Abstract

Equine infectious anemia virus (EIAV) is a lentivirus causing a persistent infection in horses characterized by recurrent febrile episodes and high levels of viremia associated with a novel antigenic strain of the virus. The virus contains two envelope glycoproteins, gp90 and gp45, and four internal proteins, p26, p15, p11 and p9. Considering that the most infected horses are able to restrict EIAV replication to very low levels and that gp45 and p26 contain highly conserved epitopes among lentiviruses, it would be necessary to identify those conserved epitopes stimulating cellular and humoral responses. The aims of this study were to determine if the synthetic peptides identified as gp45 (aa 523–547) and p26 (aa 318–346) representing two highly conserved and immunodominant regions of EIA virus are recognized by PBMC and antibodies to EIAV adult mixed-breed naturally infected carrier horses, and if these peptides are able to induce immune responses in mice. Antibodies from 100% of carrier horses, evaluated by ELISA, recognized both peptides; PBMC from 80% of carrier horses, evaluated by lymphoproliferation assay, recognized, at least, one peptide. Furthermore, immunization with 100 μg of each peptide elicited humoral and cellular responses in BALB/c mice, antibodies appeared at 48 or 63 days of immunization with gp45 or p26, respectively. Although the kinetics of gp45- and p26-specific antibody responses were similar, percentage of positivity was higher for gp45. The lymphoproliferation assay, evaluated by BrdU uptake, was higher in mice immunized with gp45 or p26 than in the control group ($P < 0.05$). Based on our findings, we consider that both peptides could be included in an effective vaccine design to induce long-term immunological memory.
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Keywords: Equine infectious anemia virus; EIAV carrier horses; Lentivirus; Synthetic peptide; gp45; p26

Abbreviations: aa, amino acids; AGID, agar gel immunodiffusion; BrdU, Bromo d-Uridine; CTL, Cytotoxic T Lymphocyte; EIAV, equine infectious anemia virus; FCA, Freund's Complete Adjuvant; Fmoc, 9-fluorenylmethoxycarbonyl; gp45, glycoprotein 45; PBMC, peripheral blood mononuclear cell; PID, principal immunodominant domain; PP, percentage of positivity; p26, protein 26; SI, stimulation index; TMB, Tetra Metil Bemcidine

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1. Introduction

Equine infectious anemia virus (EIAV), a member of the Lentivirus subfamily of retroviruses, causes a persistent infection in horses characterized by recurrent febrile episodes associated with viremia, anemia and thrombocytopenia. The cyclic nature of the disease is attributed to rapid mutations in the viral envelope gene which give rise to virus variants capable of escaping host immune responses. Viremia becomes undetectable 3–4 weeks after the initial acute stage (McGuire et al., 2000; Fraser et al., 2002).

Most infected horses are able to eventually restrict EIAV replication to very low levels and remain free of clinical disease (carrier stage). Nonetheless, these individuals do not clear EIAV, as demonstrated by the recurrence of viremia and acute signs of infection when they are immunocompromised (Fraser et al., 2002).

Control of the viremic episode requires immune responses. The initial viremia episode is probably controlled by Cytotoxic T Lymphocytes (CTL) before the appearance of the neutralizing antibody, declining to low levels when plasma viremia becomes undetectable. T helper and T cytotoxic memory cells are stimulated in response to envelope (Env) and internal structural (Gag) protein epitopes (Hammond et al., 1997; Zhang et al., 1998; McGuire et al., 2002).

The virus contains two envelope glycoproteins, gp90 and gp45, and four major non-glycosylated internal proteins, referred to as p26, p15, p11 and p9 (Hussain et al., 1988). The envelope glycoproteins and the major core proteins (p26) are the primary immunogens during a persistent infection in horses (Chong et al., 1991a).

Biochemical and immunological studies of the predominant EIA viral population, recovered during the disease have shown that conservation of epitopes appears to be a prominent property of gp45 and p26 (McGuire et al., 2000; Belshan et al., 2001).

P26 is highly conserved among not only EIAV isolates but also among isolates of puma lentivirus, feline immunodeficiency virus, visna virus, caprine arthritis encephalitis virus, HIV-1 and simian immunodeficiency virus (Chong et al., 1991a; McGuire et al., 2000; Fraser et al., 2002). Gp45 has a principal immunodominant domain between neighboring cysteine residues, which constitutes a highly conserved epitope in most retroviruses, with a potential loop structure (Chong et al., 1991b).

Lonning et al. (1999) observed that 197–309 amino acids (aa) sequence, from p26, was an area where multiple Th lymphocyte epitopes were recognized by EIAV-infected horses. In addition, Fraser et al., 2002 identified Th1 lymphocyte epitopes, localized within the same region. Major B lymphocyte epitopes of EIAV p26 are located within the carboxy-terminal region of p26 spanning 277–352 (Chong et al., 1991a). This region of the protein also has multiple reactive epitopes, which are able to cause proliferation of peripheral blood mononuclear cells (PBMC) and are recognized by CTL from EIAV-infected horses (Chong et al., 1991a; Zhang et al., 1998; McGuire et al., 2000, 2002). Evidence suggests that cellular immune responses involving antibody-dependent cellular cytotoxicity may be mediated by core-specific antibodies (Zhang et al., 1998).

The transmembrane gp45 has a principal immunodominant domain (PID), highly conserved, R32 (522–534) and R51 (534–547). Chong et al. (1991b) demonstrated that each epitope represents an antigenic determinant among diverse lentiviruses present in nature. By analyzing the antigenic characteristic of synthetic peptides between residues 523–547 of gp45, Soutullo et al. (2001) observed that 92% of naturally infected horses had antibodies capable of recognizing that sequence. Furthermore, in feline immunodeficiency virus, Pancino et al. (1995) observed a B cell epitope in the aa sequence 536–534.

Given the rapid antigenic variation of lentiviruses, it would be necessary to identify those conserved epitopes that stimulate CD4 T lymphocytes responses, promoting a strong CTL expansion and helping to maintain memory CTL horses with dissimilar MHC molecules.

The ability of horses to restrict EIAV replication to very low levels and to remain free of clinical disease during the carrier stage provides an opportunity to determine the immunological mechanisms involved in lentivirus control. Based on these findings and on the fact that there is little information on immune response in naturally infected animals, the aims of this study were to determine if two synthetic peptides, gp45 and p26, from conserved sequences of EIAV proteins are recognized by PBMC and antibodies from EIAV naturally infected carrier horses, and if these peptides

are able to induce humoral and cellular responses in mice.

2. Materials and methods

2.1. Peptide synthesis and characterization

The peptides employed in this work, identified as gp45 ERQQVEETFNLIGCIERTHVFCHTG (aa 523–547) and p26 ANEECRNAMRHLRPEDTLEEK-MYACRDIG (aa 319–346), represent two highly conserved and immunodominant regions of EIA virus.

Both peptides were synthesized manually on solid phase using 9-fluorenylmethoxycarbonyl (Fmoc) chemical strategies and following standard protocols (Atherton and Sheppard, 1989; Fields and Noble, 1990), by the Departamento de Química Orgánica of the Facultad de Bioquimica y Ciencias Biológicas. Peptides, used in the cyclic form, were prepared by oxidation of the Cys residues with I_2 solution, using peptide concentrations of 0.1 mg/ml in amonium bicarbonate buffer 0.03 M, pH 7.9 to minimize unwanted dimerization and oligomerization. The reactions were monitored by the Ellman colorimetric assay, and at the same time by HPLC (Ellman, 1959).

2.2. Horses

2.2.1. EIAV-infected carrier horses

Thirty adult mixed-breed horses, naturally infected with uncharacterized EIAV, were included in antibody assays and five of them in stimulation assays. All horses had antibodies to EIAV as determined by agar gel immunodiffusion (AGID) test (Coggins et al., 1972).

2.2.2. Control horses

Thirty healthy adult mixed-breed horses negative to the AGID test were included as uninfected controls. These animals come from a racehorse-breeding ranch with 10-year absence of clinical and serological disease checked twice annually (two annual controls to the total of the population). All of them were included in antibody and three in stimulation assays. Blood samples were collected by jugular venipuncture and serum samples were stored at −20 °C.

2.3. Mice

Females BALB/c mice of about 3 months of age were employed. Groups of five BALB/c mice were inoculated subcutaneously every 15 days for 3 months, with 200 μl of PBS containing 0 μg (group 1); 20 μg (group 2) or 100 μg (group 3) of peptides emulsified with Freund's Complete Adjuvant (FCA) (Sigma). Blood was collected by puncture of the retro orbital plexus before each immunization and serum was stored at −20 °C.

2.4. Enzyme-linked immunosorbant assay (ELISA)

Anti-gp45 and anti-p26 were determined following Engvall and Perlmann (1972), with some modifications (Soutullo et al., 2001). Microtiter plates with 96-flat-bottomed wells were coated at 4 °C with 0.5 μg of the corresponding peptide. Then, unsaturated binding sites were blocked with 3% of no-fat milk in PBS and incubated overnight at 4 °C. After washing with PBS containing Tween 20 (0.065%), the sera (50 μl of a 1/100 dilution) were added and incubated at 37 °C for 1 h. The antibody fixed was determined by adding the peroxidase conjugated rabbit serum specific for mouse IgG or equine IgG, depending on the antibody evaluated. The wells were washed three times and 50 μl of substrate-Tetra Metil Bemcidine (TMB) (1 mg/ml) in citrate buffer pH 4.0 with $H_2 0_2$ (0.05%) was added. After 5 min of incubation at room temperature, the reaction was stopped by adding 50 μl of 2.5 M H_2SO_4. The plates were read on a Multiskan Automatic ELISA Plate Reader (Labsystem) at 450 nm. Results were expressed as a percentage of a high positive reference standard, calculated as:

$$\text{PP}\,(\%) = \frac{100 \times \text{OD}\,(\text{sample}) - \text{OD}\,(\text{NCS})}{\text{OD}\,(\text{RSPC}) - \text{OD}\,(\text{NCS})}$$

where PP is the percentage of positivity; for equine, the RSPC is the reference standard positive control, a equine gammaglobulin obtained from a naturally infected horse of a farm areas with high prevalence of EIAV infection and NCS is the negative control sera from horses of a farm without preliminary reports of infection over the last 15 years. For mice, RSPC is the

reference standard positive control from mice inoculated with both purified proteins, gp45 and p26 and NCS is the negative control sera from mice prior inoculation.

2.5. BALB/c mice spleen cells isolation

Spleen cells, obtained from the animals sacrificed after 7 days of the last inoculation, were removed under sterile conditions and minced into small pieces. A small portion was disrupted through a stainless-steel mesh. Cells were washed in PBS and centrifuged through Histopaque-1077 (Amersham).

2.6. Lymphoproliferation assays

BALB/c spleen cells or PBMC obtained from blood of uninfected or EIAV-infected horses by centrifugation through Histopaque-1077 (Sigma, St. Louis, MO) were analyzed for recognition of gp45 or p26 synthetic peptide. PBMC (2×10^5) were added to each well of 96-well round-bottomed microplaques and incubated for 6 days at 37 °C in 5% CO_2 with PHA (0.5 μl) or purified p26, gp45 peptides (5 μg/ml), in 100 μl of complete RPMI medium supplemented with 50 UI/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine and 10% fetal bovine serum.

T-cell proliferation was determined by a colorimetric immunoassay, based on the measurement of Bromo d-Uridine (BrdU) incorporation during DNA synthesis. The BrdU ELISA was performed following manufacturer's instructions (cell proliferation ELISA SYSTEM. Pharmacia Biotrak). Briefly, cells were pulsed with 10 μl/well of 100 μM BrdU solution during the last 24 h of stimulation. Five days after the initial stimulation, plates were centrifuged, cells denatured with FixDenat solution and incubated for 120 min with mouse anti-BrdU mAb conjugated to peroxidase (1:100). Antibody conjugated 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 $SI = (S - C)/C$; where SI is the stimulation index and S and C represent the absorbance values for the stimulated and non-stimulated cells (control), respectively.

2.7. Statistical analyses

Student's and Kruskal–Wallis tests were used to compare specific antibody responses from horses and mice groups, respectively. Mann–Whitney U-test for independent samples was used to compare data from the proliferation assay in both animal species analyzed. Statistical significance of differences was defined at $P < 0.05$.

3. Results

Two synthetic peptides, gp45 and p26, from conserved sequences of EIAV proteins were used to evaluate the immune cellular and humoral responses elicited in horses naturally infected with EIAV during the inapparent carrier stage. The immunogenicity of both peptides was also analyzed in BALB/c mice.

3.1. Antibodies from EIAV-infected carrier horses recognize gp45 and p26 synthetic peptides

We analyzed gp45 or p26 peptide specificity of antibody elicited by EIAV-infected horses ($n = 30$) by ELISA. All horses had antibodies that were able to recognize both synthetic peptides. No reactivity was observed when we analyzed sera from EIAV-uninfected horses ($n = 30$). Results, expressed as percentage of positivity (PP), are shown in Fig. 1.

3.2. PBMC from EIAV-infected carrier horses proliferate in response to both gp45 and p26 synthetic peptides

To determine if PBMC from EIAV-infected carrier horses recognized epitopes on synthetic gp45 and/or p26 peptides, PBMC from EIAV-infected ($n = 5$) and from uninfected horses ($n = 3$) were incubated with 0.5 μg of each peptide. The proliferative response, evaluated by BrU uptake, showed that SI was higher than 2 in four of five EIAV-infected horses. Indeed, PBMC from H1, H2 and H3 recognized gp45 peptide, and PBMC from H1, H2 and H5 recognized p26. The

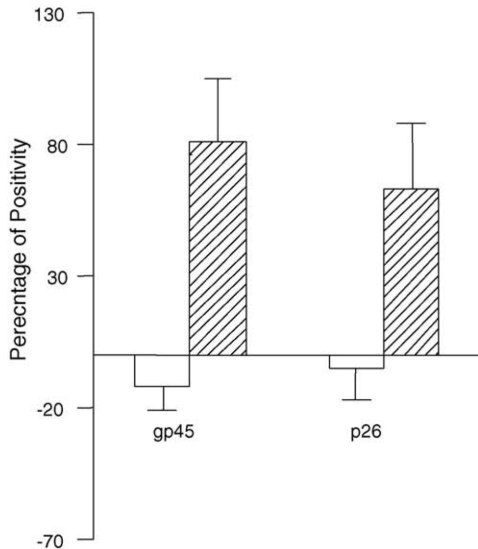

Fig. 1. Anti-gp45 and anti-p26 activity measured by ELISA and expressed as percentage of positivity (PP) in EIAV-infected (▨) and uninfected horses (□). Bars represent the means ± S.E.M. ($P < 0.001$; $n = 30$).

immunological status of horses was tested by lymphoproliferation assay using PHA. SI in both infected and uninfected horses was higher than 10. The only horse that did not recognize any peptide (H4) had a low SI (3.6) for PHA stimulated. Results are shown in Fig. 2.

3.3. Synthetic gp45 and p26 peptide stimulate both humoral and cellular immune responses in mice

In order to evaluate the ability of both synthetic peptides gp45 and p26 to induce humoral and cellular immune responses, three groups of mice were inoculated for 3 months, with 0 (group 1), 20 (group 2) or 100 μg (group 3) of each peptide emulsified with FCA.

The antibody synthesis was evaluated in serum samples, collected 7 days after each inoculation, and tested for IgG anti-gp45 or anti-p26 peptides by ELISA.

As shown in Fig. 3a and b, there was a significantly higher level of antibodies when 100 μg of peptide was used ($P < 0.05$). This response appeared at 48 days of immunization with peptide gp45 and at 63 days of immunization with p26. Although the kinetics of gp45- and p26-specific antibody responses was similar, the levels of PP of gp45 were greater than PP of p26. Mice immunized with 20 μg of either peptide did not elicit antibody response.

In order to analyze cellular immune responses, spleen cells obtained from the mice sacrificed after 7 days of the last inoculation were incubated with gp45, p26 or PHA. As shown in Fig. 4, the lymphoproliferation assay, evaluated by BrdU uptake after 6 days of culture and expressed as SI, was higher in mice

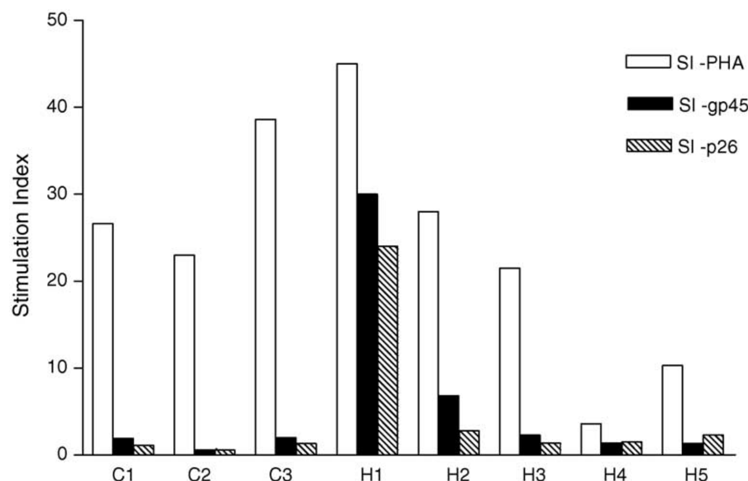

Fig. 2. Determination of BrdU uptake by PBMC stimulated with p26 or gp45 peptides and with PHA, from individual horses (H1–H5) expressed as SI. No EIAV-specific lymphoproliferation was detected in the PBMC of uninfected horses (C1–C3).

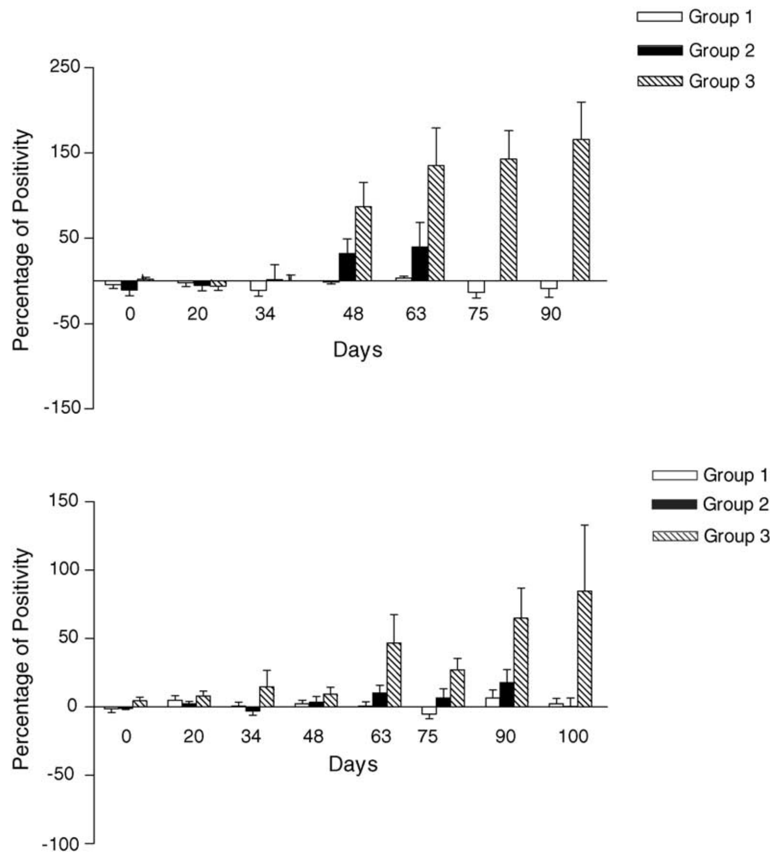

Fig. 3. (a) Anti-gp45 activity was evaluated by ELISA in serum samples obtained from mice immunized with 0 (group 1), 20 (group 2) or 100 (group 3) μg of the peptide. Antibody levels are expressed as percentage of positivity (PP). Bars represent means ± S.E.M. ($P < 0.05$; $n = 5$). (b) Anti-p26 activity was evaluated by ELISA in serum samples obtained from mice immunized with 0 (group 1), 20 (group 2) or 100 (group 3) μg of the peptide. Antibody levels were expressed as percentage of positivity (PP). Bars represent the means ± S.E.M. ($P < 0.05$; $n = 5$).

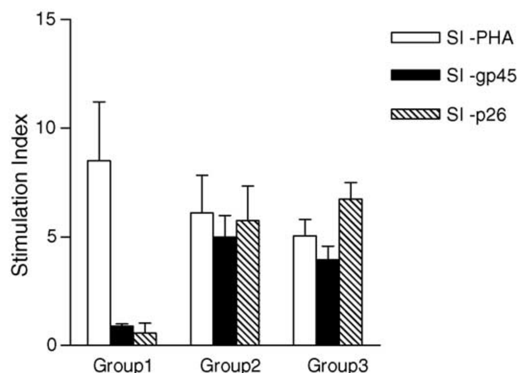

Fig. 4. SI was evaluated by the lymphoproliferation assay of spleen cells of BALB/c mice immunized with 0 (group 1), 20 (group 2) or 100 μg (group 3) of the gp45 or p26 peptides. The cells were incubated with gp45, p26 or PHA. Bars represent the means ± S.E.M. ($P < 0.05$; $n = 5$).

immunized with gp45 or p26 than in the control group ($P < 0.05$).

4. Discussion

The ability of horses to restrict EIAV replication to very low levels and to remain free of clinical disease provides an opportunity to study the immunological mechanism involved in this lentivirus control. The generation of humoral and cellular responses is critical to the control of viral infections. Kono et al. (1976) observed that viremic febrile episode is induced after administration of immunosuppressants to clinical asymptomatic EIAV carrier horses. Given the antigenic variation occurring in each febrile episode, identification of conserved epitopes from envelope

glycoprotein (gp45) and capsid protein (p26), which are able to stimulate memory lymphocytes during the carrier stage, and which also have immunogenic properties, would be important for the understanding of in vivo immune responses that contribute to EIAV control. Those epitopes should be considered for the design of an efficacious vaccine that protects animals from possible infection.

In this study, we have observed that two synthetic peptides that mimic conserved regions of EIAV, corresponding to the sequence of aa 318–346 and 523–547 of p26 and gp45, respectively, can be recognized by PBMC and by antibodies from naturally infected horses during the carrier stage. In addition, we have shown that both peptides generate strong immune cellular and humoral responses in mice.

Concerning the first assertion, antibodies from 100% of carrier horses analyzed recognized both gp45 and p26 peptides, and PBMC from three of five infected horses recognized gp45 or p26 peptides. These results might suggest that both peptides would be presented by dissimilar MHC alleles, since, although the molecules of MHC have not been typified, the horses studied were mixed-breed. Our results partially agree with findings for experimentally infected carrier horses. McGuire et al. (2000) found that three overlapping peptides p26-16 (314–333), p26-17 (326–345) and p26-18 (338–359) were each reactive with PBMC from at least four of five EIAV-infected horses. Recently, Chun et al. (2004) observed that CTL from 50% of EIAV-infected carrier horses with diverse MHC alleles recognized two epitope clusters, identified as EC3 and EC4, corresponding to 281–313 and 317–356, respectively. The latter includes p26 peptide studied in this work. Chong et al. (1991b) observed that the region 276–359 was recognized by antibodies from all experimental or naturally infected horses analyzed.

Regarding gp45 peptide, on the basis of serological study, Chong et al. (1991a) demonstrated that the residues 522–547 would react strongly with most sera from persistently infected horses. McGuire et al. (2000) suggested that residues 520–637 (T3b) and 632–736 (T3c) would contain epitopes capable of stimulating CTL from two of seven EIAV-infected horses.

Finally, the immunogenicity of both peptides was analyzed in BALB/c mice. The results show that immunization with free unconjugated gp45 or p26 peptides, induced humoral and cellular responses after three to four inoculations, despite their low molecular weight.

It is commonly asumed that antigens with a molecular weight smaller than 2–5 $\times$ 10^3 Da should be conjugated to a carrier molecule for eliciting an immune response (Van Regenmortel and Muller, 1999). However, the interaction of the peptide with the heterogeneous carrier surface could possibly alter the peptide original configuration or induce the wrong conformation, thus altering the peptide immunogenicity.

Furthermore, immunization with cyclized peptides has been found to induce the production of antibodies possessing a high degree of cross-reactivity with the intact protein. In preliminary assays, we observed that antibodies anti-gp45 peptide were able to recognize EIAV-infected macrophages from horses with acute disease (data not shown).

Different vaccination strategies against EIAV infection have been studied. Vaccines based on inactivated whole viruses or on purified viral envelope glycoproteins elicit sterile protection only from homologous EIAV challenge and immunization with a recombinant envelope glycoprotein sub-unit vaccine results in rapid and severe enhancement of clinical disease symptoms accompanied by accelerated virus replication in about 40% of vaccinated ponies (Issel et al., 1992; Raabe et al., 1998; Hammond et al., 1999).

Alternatively, epitope-based vaccines may represent an attractive design because they are able to focalize immune responses. Several approaches have been made with synthetic peptides against viral diseases (Langveld et al., 1994; Hamajima et al., 1995; Elliott et al., 1999; Volpina et al., 1999; Huerta et al., 2002; Ulh et al., 2002; Wang et al., 2002).

Ridgely et al. (2003) evaluated the efficacy of EIAV lipopeptide immunization with a CTL epitope from surface unit of EIAV, but they could not prevent infection although it provided a significant protective effect against clinical disease following virus challenge.

Based on our findings, we consider that both peptides could be included in an effective vaccine design to induce long-term immunological memory.

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