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Application of synthetic peptides to the diagnosis of neurocysticercosis

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Summary We tested the possible diagnostic utility of five *Taenia saginata* oncosphere-derived synthetic peptides in *T. solium* neurocysticercosis (NC). The five peptides correspond to protein sequences with high antigenic indexes that were cloned from a *T. saginata* oncosphere cDNA library. The test samples consisted of cerebrospinal fluid (CSF) samples randomly collected from patients referred from Mexican and Brazilian neurological institutes. Indirect enzyme-linked immunosorbent assays (ELISA) were carried out with the peptides either unconjugated or coupled to carrier proteins, and were compared with results obtained using *T. solium* cyst fluid as a positive control. For active inflammatory NC, the higher sensibility (93%) and specificity (85%) was obtained with peptides HP6-2 and Ts45W-1, respectively, coupled to ovalbumin, in both Mexican and Brazilian patients. Examining the results of the individual peptide assays in combination, in some instances, improved the sensitivity to 100%.

keywords peptides, *Taenia solium*, neurocysticercosis, diagnosis

Introduction

Neurocysticercosis (NC) is the most common worldwide parasitic disease of the central nervous system and its prevalence is highest in developing countries (Del Brutto *et al.* 1998). In recent years, human cases of NC have emerged in developed countries, such as the USA and Europe, mostly because of migrant workers (Shandera *et al.* 1994; Rousseau *et al.* 1999; Carangelo *et al.* 2001; Terraza *et al.* 2001). Accurate diagnosis of NC still requires cranial imaging technology such as computerized tomography (CT) and/or magnetic resonance imaging (MRI), techniques that are expensive and frequently inaccessible to people of endemic areas (Sciutto *et al.* 2000). The diagnosis of NC without imaging studies is difficult because of the highly variable clinical symptoms, ranging from asymptomatic to a very severe neurological syndrome with intracranial hypertension or dementia, with a variety of non-specific mild clinical symptoms in between.

For immunological diagnosis, the enzyme-linked immunosorbent assay (ELISA) and the enzyme-linked immunotransfer blot (EITB) are the most widely used techniques (Rosas *et al.* 1986; García *et al.* 1995). Although the initial data suggested that the EITB using parasite glycoproteins isolated by lectin-affinity chromatography was highly specific and sensitive (Tsang *et al.* 1989), results from subsequent studies were less encouraging (Ordoñez *et al.* 1996). One of the major disadvantages of almost all reported immunodiagnostic methods is the poor reproducibility between different laboratories, presumably because of differences in the clinical status of patients examined and variability in the nature and purity of the parasite antigen extracts

employed. There is therefore an inherent advantage of reproducibility in diagnostic tests based on recombinant proteins or synthetic peptides (Gevorkian *et al.* 1996; Hernández *et al.* 2000; Ferrer *et al.* 2003). Such a permanent source of diagnostic reagents would also be useful in traditionally non-endemic countries at risk of sporadic incursions of the parasites, or where the collection of *T. solium* cysticerci is difficult.

Recently, synthetic peptides based on antigenic sequences identified in immunogentic oncosphere proteins of *Taenia saginata* have been shown to function well in the diagnosis of bovine cysticercosis (Ferrer *et al.* 2003), and hence we evaluated them for the diagnosis of human NC using samples from Mexico and Brazil. The five peptides consist of: one peptide (HP6-3) derived from the sequence of the 18 kDa *T. saginata* surface/secreted oncospheral adhesion antigen identified by McAb-HP6, two peptides (Ts45W-1 and Ts45W-5) derived from the sequence of the *T. saginata* homologue of the *T. ovis* 45W protective gene family, one peptide (TS45S-10) derived from a *T. saginata* sequence with significant similarity to the *T. ovis* 45S protective antigen, and one peptide (TEG-1) derived from the sequence of the *T. saginata* homologue of *Echinococcus* spp. main surface protein. The corresponding proteins, which may also be potential protective antigens, consist of: the 18 kDa *T. saginata* surface/secreted oncospheral adhesion antigen identified by McAb-HP6 (Benítez *et al.* 1996; Bonay *et al.* 2002; Ferrer *et al.* 2003), the *T. saginata* homologue of the *T. ovis* 45W protective gene family (Johnson *et al.* 1989; Waterkeyn *et al.* 1995; Ferrer *et al.* 2003), the *T. saginata* homologue of *T. ovis* 45S protective antigen (Waterkeyn *et al.* 1995; Ferrer *et al.* 2003) and the *T. saginata* homologue of *Echinococcus* spp. surface protein (Benítez *et al.* 1998).

Material and methods

Cerebrospinal fluid

Cerebrospinal fluid (CSF) samples were collected in Mexico and Brazil. The Mexican samples formed three clinically distinct groups: 27 CSF from patients (12 men and 15 women, mean age 40 ± 13 years) with radiologically diagnosed NC taken before any treatment, 16 CSF from patients with clinically diagnosed NC taken 1–12 months after specific drug treatment (albendazole +prednisona) and 31 CSF from non-NC control patients. We also collected and tested 11 CSF of the 27 NC patients 1–8 months after cysticidal treatment (albendazole + prednisona).

The CSF samples from Brazil were obtained by suboccipital puncture at the Neurological Department of the Medicine, University of Botucatu and consisted of 24 CSF samples from control, non-NC neurological patients and 32 from diagnosed NC patients (24 men and eight women, mean age 38.6 ± 9.4 years), 1–360 days after drug treatment (albendazole).

Clinical details and characteristics of each CSF sample, including the number of cells, were obtained from the medical records. The diagnosis of NC, as determined by neuroimaging, was classified as 'active' when at least one viable cysticercus or arachnoiditis was present, and cases were classified as either single- or multiple-viable cysts. Alternatively, when neuroimaging studies only detected calcifications or meningeal fibrosis, which are the permanent sequelae of previous infections (Sotelo *et al.* 1985), the disease was classified as 'inactive'. Active cysticercosis patients were further classified as 'inflammatory' or 'non-inflammatory' by counting mononuclear cells in their CSF; inflammatory being defined as having more than six mononuclear cells per cubic millimetre. Controls were patients for whom the diagnosis of NC had been excluded by neuroimaging, and who were seen in neurological institutes for inflammatory, vascular, infectious and degenerative disease.

Peptides

The peptides (Ferrer *et al.* 2003) examined in this study and described in the introduction are summarized in Table 1. Peptides were synthesized by stepwise solid-phase synthesis with N^{α}-tert-butyloxycarbonyl (BOC) derivatives of L-amino acids on phenyl-acetamidomethyl (PAM) resin (Sigma Chemical Co, St Louis, MO). The peptides were 95% pure as judged by high-performance liquid chromatography (HPLC) on analytical C_{18} reversed-phase column (3.9 × 150 mm; Delta Pak, Waters). The correct amino acid sequence of each peptide was confirmed by protein sequencing on a pulsed liquid-phase protein sequencer (Applied Biosystems) at the National Institute of Cardiology, Mexico City. Peptides were coupled to maleimide activated ovalbumin (OVA) and keyhole limpet haemocyanin (KLH) (Pierce Warriner Ltd.) following the manufacturer's instructions.

Antibody detection by ELISA

Cerebrospinal fluid antibody titres were assessed by indirect ELISA using either *T. solium* cyst fluid or the peptides as antigens as previously described (Ramos-Kuri *et al.* 1992). Plates were set up with duplicate sample determinations. The assays were routinely repeated to

Table 1 The amino acid sequences and Genebank numbers of the peptides selected for study

Protein description and Genebank numbers	Sequence designation	Peptide designation	Number of amino acids	Amino acid sequence
Major secreted 'functional' antigen identified by McAb HP6 (Genebank No. X95983)	HP6	HP6-3	15	CRFVKTPSKKKTKSR
Taenia ovis 45W gene family homologue (Genebank no. AJ430567)	Ts45W	Ts45W-1	24	AYEQPIERTVVGHQTLRDIFVWGC
		Ts45W-5	10	CGGTEESVVTASRS
Taenia ovis 45S homologue (Genebank no. AJ430566)	Ts45S	Ts45S 10	14	CGGTEESVVTASRS
Echinococcus surface protein homologue (Genebank no. X97000)	TEG	TEG-1	23	CRDPSKMRDIDRHHEYNVREGND

Table 2 Range of age of Mexican and Brazilian drug-treated patients with different clinical NC

	Mexico		Brazil		
Range of age (years)	Active inflammatory	Active non-inflammatory	Active inflammatory	Active non-inflammatory	Inactive
0–10	0	0	0	0	0
11–20	1	0	0	0	2
21–30	1	4	1	0	3
31–40	6	2	2	3	2
41–50	6	1	17	0	1
51–60	3	1	0	0	0
>60	1	1	0	0	1

Table 3 Sensitivity of ELISA in CSF using VFA or different free synthetic peptides as a source of antigen (Mexican and Brazilian drug-treated patients)

	Mexico		Brazil		
	Active non-inflammatory ($n = 8$)	Active inflammatory ($n = 8$)	Active non-inflammatory ($n = 3$)	Active inflammatory ($n = 9$)	Inactive ($n = 7$)
Vesicular fluid	87.5 (0.88 ± 0.8)	100 (1.3 ± 0.58)	100 (0.68 ± 0.24)	100 (1.28 ± 0.34)	57 (0.42 ± 0.5)
Ts45W-1	12.5 (0.13 ± 0.08)	62.5 (0.34 ± 0.55)	0 (0.09 ± 0.007)	77.8 (0.71 ± 0.45)	0 (0.086 ± 0.013)
Ts45W-5	12.5 (0.12 ± 0.08)	50 (0.34 ± 0.5)	0 (0.12 ± 0.02)	77.8 (0.72 ± 0.55)	0 (0.094 ± 0.02)
HP6-3	12.5 (0.19 ± 0.08)	50 (0.38 ± 0.43)	0 (0.18 ± 0.02)	77.8 (0.84±0.58)	0 (0.14 ± 0.03)
TEG-1	12.5 (0.11 ± 0.05)	50 (0.29 ± 0.45)	0 (0.1 ± 0.04)	77.8 (0.5 ± 0.45)	0 (0.09 ± 0.02)
Ts45S 10	12.5 (0.11 ± 0.09)	62.5 (0.26 ± 0.27)	0 (0.13 ± 0.01)	77.8 (0.63 ± 0.49)	0 (0.11 ± 0.02)

The values are sensitivity values and those in parentheses are OD values (mean ± SD).
VFA, vesicular fluid antigen.

check for reproducibility. A sample was considered positive if the specific optical density (OD) values were greater than the mean of the values obtained with negative CSF samples plus two standard deviations (SD). Five to 10 negative CSF from confirmed non-NC neurological patients different from those included in the study to determine the specificity were used in each plate to determine the cut-off value.

Statistical analysis

Sensitivity was calculated as the number of proven NC cases that were ELISA positive, and specificity as the number of negative control samples that were ELISA negative. Dichotomous data were compared using the chi-square Pearson test or the Yates corrected chi-square test. Differences in mean values between groups were

Table 4 Sensitivity (%) of ELISA using free unconjugated peptides or coupled with carrier proteins (Brazilian CSF samples from eight treated patients)

	Free unconjugated peptide	KLH	OVA
Ts45W-1	75	75	87.5
Ts45W-5	75	75	87.5
HP6-3	87.5	75	87.5
TEG-1	75	75	87.5
Ts45S 10	75	75	87.5

Sensitivity = number of positives/number of sample tested.

analysed by Student's *t*-test or by a non-parametric test (Mann–Whitney).

Results

The five peptides were first tested in a free unconjugated form and the most obvious conclusion is that the peptides function best for antibody detection in cases of active inflammatory NC (Table 3), especially in patients aged 30–50 years (Table 2).

As different peptides do not necessarily bind with equal efficiencies to ELISA plates, as an attempt to standardize conditions, the five peptides were coupled to protein carriers, OVA and KLH. These preliminary experiments indicated that there was no significant difference in the results obtained using free simple peptides or peptides coupled to the carrier proteins (Table 4).

These peptide carrier protein conjugates were used as probes for the Mexican and Brazilian CSF samples. Employing Mexican active inflammatory NC, the sensitivity using Ts45W-5 and HP6-3 coupled to KLH or Ts45W-1, HP6-3, TEG-1 and Ts45S coupled to OVA was as good as that obtained using cyst fluid antigen. Similarly, with the Mexican active non-inflammatory NC, peptide HP6-3 OVA gave the same sensitivity as cyst fluid antigen (Table 5). Finally, using Ts45W-1, Ts45W-5 and HP6-3 coupled to OVA with the Brazilian active inflammatory NC, the same sensitivity was obtained as with cyst fluid antigen (Table 5).

As immune responses to parasites are characteristically highly heterogeneous, the effect of combining the results from certain individual peptide assays was examined. With the Mexican samples, a combination of HP6-3 OVA and Ts45W-1 OVA gave a sensitivity and specificity quite similar to those obtained with cyst fluid (Tables 5 and 6). Similarly, with the Brazilian samples, combinations of Ts45W-5 OVA and Ts45S OVA or TEG-1 OVA also indicate an improved sensitivity (Table 5) with a good specificity (Table 6), which were between 93% and 100%.

Table 5 Sensitivity of ELISA in CSF using VFA or different synthetic peptides coupled with carrier proteins as a source of antigen

	Mexican NC		Brazilian NC		
	Active non-inflammatory ($n = 9$)	Active inflammatory ($n = 18$)	Active non-inflammatory ($n = 3$)	Active inflammatory ($n = 20$)	Inactive ($n = 9$)
Vesicular fluid	100 (0.64 ± 0.28)	100 (2.13 ± 0.4)	100 (1.03 ± 0.9)	100 (1.55 ± 0.37)	55.5 (0.43 ± 0.57)
Ts45W-1 KLH	0 (0.29 ± 0.08)	72.2 (0.68 ± 0.28)	0 (0.24 ± 0.02)	50 (0.53 ± 0.38)	0 (0.23 ± 0.03)
Ts45W-5 KLH	0 (0.28 ± 0.09)	88.9† (0.66 ± 0.31)*	33.3 (0.1 ± 0.07)	50 (0.75 ± 0.89)	0 (0.09 ± 0.02)
HP6-3 KLH	44.4 (0.75 ± 0.34)	77.8 (1.25 ± 0.69)*	0 (0.27 ± 0.09)	45 (0.79 ± 0.86)	0 (0.22 ± 0.07)
TEG-1 KLH	11.1 (0.3 ± 0.07)	66.7 (0.6 ± 0.26)	0 (0.17 ± 0.05)	65 (0.80 ± 0.93)	0 (0.15 ± 0.04)
Ts45S 10 KLH	11.1 (0.26 ± 0.07)	66.7 (0.41 ± 0.21)	33.3 (0.17 ± 0.03)	60 (0.62 ± 0.75)	0 (0.12 ± 0.03)
Ts45W-1 OVA	22.2 (0.21 ± 0.08)	88.9 (0.76 ± 0.42)*	33.3 (0.2 ± 0.01)	85 (0.93 ± 0.87)*	0 (0.16 ± 0.05)
Ts45W-5 OVA	11.1 (0.23 ± 0.09)	66.7 (0.8 ± 0.58)	33.3 (0.17 ± 0.007)	90 (0.98 ± 0.85)*	11.1 (0.14 ± 0.03)
HP6-3 OVA	77.7 (0.88 ± 0.44)*	83.3 (1.44 ± 0.64)*	33.3 (0.5 ± 0.1)	85 (0.97 ± 0.75)*	0 (0.33 ± 0.14)
TEG-1 OVA	55.5 (0.18 ± 0.07)	83.3 (0.47 ± 0.38)*	0 (0.09 ± 0.006)	70 (0.25 ± 0.27)	0 (0.09 ± 0.04)
Ts45S 10 OVA	33.3 (0.23 ± 0.08)	77.8 (0.8 ± 0.55)*	0 (0.09 ± 0.007)	75 (0.4 ± 0.54)	11.1 (0.08 ± 0.02)
HP6-3 OVA + Ts45W-1 OVA	77.7*	100*			
Ts45W-5 OVA + Ts45S 10 OVA or TEG-1 OVA			33.3	95*	11.1

Values are sensitivity values and those provided in parentheses are OD values (mean ± SD).
* No significant differences with VFA.

Table 6 Specificity (%) of ELISA in CSF using VFA or different synthetic peptides coupled with carrier protein as a source of antigen (Mexican and Brazilian subjects)

	Neurological patients (No NC)	
	Mexican ($n = 31$)	Brazilian ($n = 24$)
Vesicular fluid	96.8 (0.11 ± 0.05)	95.8 (0.08 ± 0.03)
Ts45W-1 KLH	96.8 (0.16 ± 0.04)	100 (0.25 ± 0.04)
Ts45W-5 KLH	93.5 (0.15 ± 0.04)	94.4 (0.1 ± 0.06)
HP6-3 KLH	93.5 (0.13 ± 0.03)	95.8 (0.25 ± 0.1)
TEG-1 KLH	100 (0.14 ± 0.04)	95.8 (0.18 ± 0.13)
Ts45S 10 KLH	96.8 (0.14 ± 0.04)	95.8 (0.13 ± 0.07)
Ts45W-1 OVA	93.5 (0.18 ± 0.06)	91.7 (0.16 ± 0.1)
Ts45W-5 OVA	96.8 (0.18 ± 0.05)	91.7 (0.16 ± 0.1)
HP6-3 OVA	93.5 (0.17 ± 0.05)	100 (0.38 ± 0.16)
TEG-1 OVA	96.8 (0.22 ± 0.1)	95.8 (0.08 ± 0.04)
Ts45S 10 OVA	93.5 (0.2 ± 0.07)	95.8 (0.1 ± 0.08)
HP6-3 OVA + Ts45W-1 OVA	90.3	
Ts45w-5 OVA + Ts45S 10 OVA or TEG-1 OVA		87.5

Values in parentheses are OD values (mean ± SD).

Table 7 Sensitivity of the antibody detection assay before and after specific treatment in 11 Mexican NC patients

	Sensitivity (%)	
	Before treatment	After treatment*
Vesicular fluid	100 (1.74 ± 0.72)	100 (1.45 ± 0.75)
Ts45W-1 KLH	45.4 (0.55 ± 0.26)	72.7 (0.58 ± 0.20)
Ts45W-5 KLH	63.6 (0.56 ± 0.31)	72.7 (0.57 ± 0.20)
HP6-3 KLH	81.8 (1.21 ± 0.58)	72.7 (1.01 ± 0.28)
TEG-1 KLH	63.6 (0.56 ± 0.26)	54.5 (0.51 ± 0.18)
Ts45S 10 KLH	63.6 (0.37 ± 0.13)	81.8 (0.38 ± 0.13)
Ts45W-1 OVA	72.7 (0.64 ± 0.43)	72.7 (0.55 ± 0.27)
Ts45W-5 OVA	45.4 (0.56 ± 0.38)	54.5 (0.53 ± 0.26)
HP6-3 OVA	81.8 (1.30 ± 0.52)	81.8 (1.05 ± 0.36)
TEG-1 OVA	81.8 (0.36 ± 0.16)	54.5 (0.33 ± 0.11)
Ts45S 10 OVA	72.7 (0.65 ± 0.41)	63.6 (0.57 ± 0.24)

* These CSF were collected 1–8 months after treatment.
Values in parentheses are OD values (mean ± SD).

Interestingly, cysticidal and corticosteroid treatment did not significantly modify CSF levels of antibodies or sensitivity of the assay employing the different peptides as a source of antigen (Table 7).

Discussion

Diagnosis of human, bovine and porcine cysticercosis still relies on the detection of antibodies using parasite extracts or multi-component glycoprotein fractions. The use of sufficiently sensitive and specific recombinant proteins or synthetic peptides as antigens in such assays would provide the much needed reproducibility and circumvent reliance on the less reproducible parasite extracts. Five peptides of high antigenic indices from the sequences of four potentially protective *T. saginata* proteins already shown to have utility in the diagnosis of *T. saginata* cysticercosis (Ferrer *et al.* 2003) were tested as possible reagents for the diagnosis of human neurocysticercosis. It is worth mentioning that the HP6-3 peptides were taken from the sequence of a oncospheral *T. saginata* antigen with proven protective capacity against bovine *T. saginata* cysticercosis (LJS Harrison and RME Parkhouse, unpublished data), and will be of interest to determine the relevance of homologous anti-HP6-3 antibodies in the human disease.

Most of the data presented was obtained with peptide–protein conjugates. As seen in Table 4 these peptides gave comparable results in soluble form or coupled to proteins (OVA or KLH). Peptides HP6-3 and Ts45W-1 coupled to OVA detected the majority of the active inflammatory NC patients [83.3% (Figure 1) and 88.9% (Figure 2) of the Mexican, respectively, and 85% of the Brazilian]. Notably, as active NC was mainly found in patients between 30 and 50 years of age, the test could be more effective in this age group. The fact that the results obtained with the Mexican samples mirror those of the Brazilian samples suggests that similar antigenic epitopes are shared by *T. solium* from these two countries. More than 45% of these patients also recognized the other four peptides that were tested, a fact that points to a low variability of cysticercal antigens and an important cross-immunity

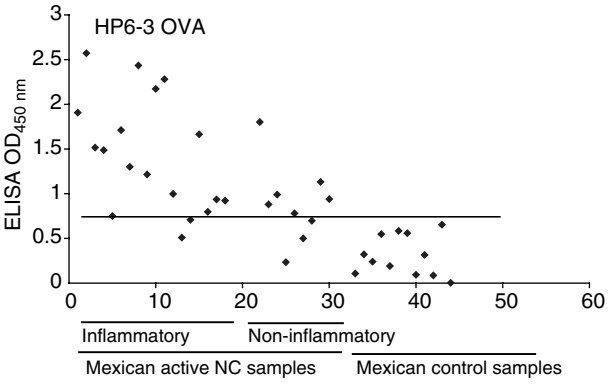

Figure 1 Representative scatter plot for HP6-3 OVA of Mexican active Neurocysticercosis patients (inflammatory and non-inflammatory) and controls. The line represents the cut-off value. A good signal to background ratio is observed between the non-NC patients and the NC patients.

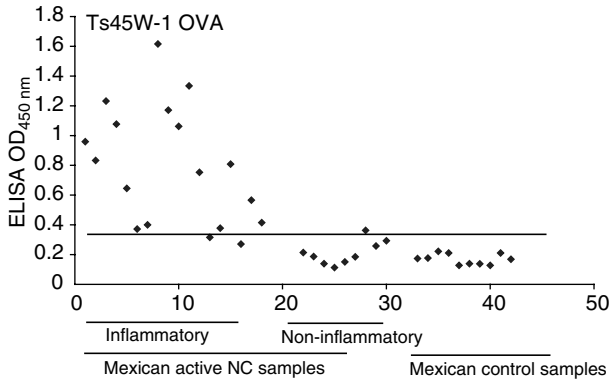

Figure 2 Representative scatter plot for Ts45W-1 of Mexican active Neurocysticercosis patients (inflammatory and non-inflammatory) and controls. The line represents the cut-off value. A good signal to background ratio is observed between the non-NC patients and the NC patients.

between cestodes. A combination of two peptides [HP6-3 (OVA) and Ts45W-1 (OVA)] reached a sensitivity of 100% and a specificity as good as that obtained using cyst fluid and thus merits serious consideration for the design of a diagnostic kit.

In summary, this study has identified peptides of potential use in NC diagnosis. Simple and reproducible antibody detection assays for NC would find utility in hospitals and in epidemiological studies in endemic areas and would also be a useful resource in non-endemic countries to determine possible exposure to the parasite.

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