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SILENT BRAIN INFARCTION IN STROKE PATIENTS: A PILOT DOUBLE-CENTER STUDY

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ABSTRACT

Silent Brain Infarcts (SBI) are associated with an increased risk of subsequent stroke. SBI are incidentally revealed by CT or MRI executed in stroke patients.

A prospective study was undertaken involving 200 consecutive patients aged >45 years with brain infarct admitted in University of Alberta Hospital, Canada (100 patients) and Valie-Asr Hospital, Iran (100 patients) in 2003. Patients were divided in age groups aged <65 and >65. All of the patients underwent brain CT. A stroke neurologist with knowledge of stroke history subsequently reviewed the scans and diagnosed SBI. The relations of race, gender and age groups with SBI were analyzed with chi-square and Fisher exact tests.

SBI were present in 26.9% of patients aged <65 and 48.6% of patients aged >65. Canadian patients were significantly more preponderant to SBI in age group> 65 (p= 0.013). The mean age of Canadian patients was significantly higher than Persians (p<0.001). Within a total of 200 patients the female gender was significantly more preponderant to SBI (p= 0.02) which was not related to age and race groups. Small vessel territory infarct was present in 87% of our patients with SBI.

SBI are common in stroke patients. The frequency of SBI is higher in female gender and the elderly.

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Keywords: Silent stroke, infarct, gender, incidence.

INTRODUCTION

Each cubic millimeter of brain is vulnerable to ischemia and infarction, but in only about a third of the brain is ischemia associated with any symptom or sign. Neurologists often neglect the importance of Silent Brain Infarcts (SBI). SBI are frequently seen on neuroimaging in healthy elderly people and sometimes incidentally revealed by CT scan executed for other purposes. SBI are 5 times as prevalent as symptomatic brain infarcts in the general population.[1] The presence of SBI increases the risk of stroke 3 fold, independently of other stroke risk factors.[2] Symptoms of SBI are not noticed or recognized by the patient. There are mainly two reasons leading to silence of the brain infarct:[3] either the size and/or location of the lesion did not lead to symptoms or the symptoms produced by the SBI have not been recognized or have been forgotten by the patient. The latter category is related to the cerebral infarcts with transient symptoms, which result from an infarct visible by neuroimaging. This pilot double-center study revealed the relations between gender, race, age groups and SBI in the Canadian and Persian stroke patients.

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MATERIAL AND METHODS

200 consecutive patients aged >45 years with brain

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infarct admitted in University of Alberta Hospital, Canada (100 patients) and Valie-Asr Hospital, Khorasan, Iran (100 patients) in 2003 were enrolled in a prospective study. Aphasia, unconsciousness, cognitive impairment and airway barrier were considered as exclusion criteria. Patients were considered in two age groups, <65 and >65. Stroke and transient ischemic attack were diagnosed by stroke neurologists according to WHO criteria.[4] The absence of signs and symptoms of previous brain ischemia was ascertained by stroke neurologists with careful examination of the patients, interviews of the patients and their relatives and checking medical records. All patients underwent CT of the brain. We defined infarcts as focal hypodense lesions compatible with vascular territories. Subcortical lesions <2cm in diameter were defined as Small Vessel Territory Infarcts (SVTI).[5] Cortical lesions and lesions >2cm diameter were considered as Large Vessel Territory Infarcts (LVTI).[5]

We defined SBI as CT evidence of 1 or more infarct lesions, without history of the corresponding stroke manifestations. Patients with previous symptomatic and silent infarcts were considered in the SBI group. A stroke neurologist with knowledge of stroke history subsequently reviewed the scans and categorized the infarct lesions as silent or symptomatic. Data on patient demographics and imaging findings were kept in a data base.

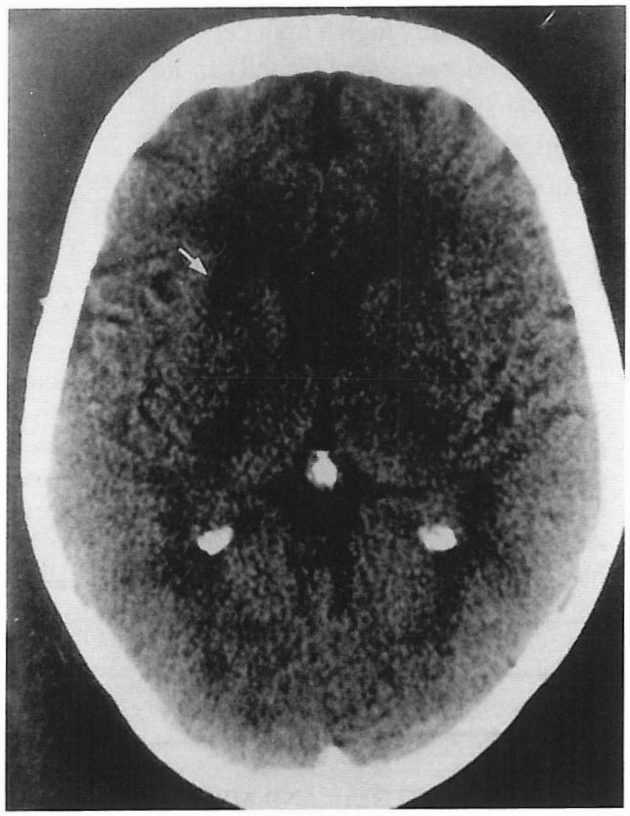

Fig.1. A silent small deep infarct in the anterior limb of the internal capsule (arrow).

The Chi-square and Fisher exact tests were used whenever appropriate in statistical analysis and $p<0.05$ was declared as significant.

RESULTS

A silent small deep infarct on CT is shown in Fig 1.

In the Canadian group 48 females and 52 males and in the Persian group 54 females and 46 males enrolled in the study. SBI were found in 86 patients (86/200; 43%). SBI were present in 14 patients in age group <65 (14/52; 26.9%) and in 72 patients in age group >65 (72/148; 48.6%). Among 86 patients with SBI, 65 patients (65/86; 75.5%) had SVTI, 11 patients (11/86; 12.8%) had LVTI and 10 patients (10/86; 11.6%) showed SBI in both territories. SBI were found in 49% of Canadian and 37% of Persian stroke patients. Canadian patients were significantly more preponderant to SBI only in age group >65 ($p= 0.013$). This finding could be due to the significantly higher mean age of Canadian patients in comparison to Persians in this age group ($p<0.001$). Within a total of 200 stroke patients, female gender was significantly more preponderant to SBI ($p= 0.02$). We did not find any statistically significant association between gender and age groups in all of the stroke patients and in each race group separately. Canadian patients were more prepond-erant to SVTI than Persian patients in age groups <65 ($p= 0.034$) and > 65 ($p= 0.049$). The female gender was significantly more preponderant to SVTI in all of the 200 patients ($p= 0.011$) and in the Canadian group ($p= 0.03$) but not in the Persian group ($p= 0.123$). Canadian patients were significantly more preponderant to LVTI in age group >65 ($p= 0.042$) but not in age group <65 ($p= 0.630$). There was not a significant female preponderance to LVTI in both age groups in Canadian and Persian stroke patients.

DISCUSSION

This pilot double-center study like most of the studies dealing with SBI[3] presents with methodological limitations. We studied patients who have already developed a second, this time a symptomatic infarct at the time of SBI diagnosis. Therefore the natural history, incidence and prevalence of SBI in our patients are not evaluated. In age group >65, Canadian patients were significantly more preponderant to SBI than Persian patients. The higher mean age of Canadian versus Persian patients could explain this finding. Although the results of our pilot study should not be interpreted to Canadian and Persian populations, however population-based studies have found that a prevalence of SBI gradually increased with age.[1,2,6] The significantly higher preponderance of female gender to SBI in our 200 patients was

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not related to age and race effects. Population-based studies have found a 30%-40% higher prevalence of SBI among women.[1,7] This is in contrast to common observations regarding symptomatic stroke that is more frequent in men.[3] This higher prevalence of SBI in women was not found in the younger cohort of the Japanese study.[8]

SVTI was present in 87% of our patients with SBI. A similar high frequency of lacunar infarcts were found in the other studies.[9,10] This finding may explain why these lesions are asymptomatic, because the majority of lacunes occur in areas such as the lentiform nucleus and are thought to be asymptomatic or at least unrecognized.

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SP Mab derived from the rat/mouse heterohybridoma NC1/34 has been used by several investigators to detect SP in different tissues.[19-21] In these experiments, we used inhibition ELISA or dot immunoassay to determine the relative affinities of α-SP MAb for SP and its fragments. Also, we examined SP and SP^{6-11} analogues with sequential substitution of alanine for each of their amino acids to identify the amino acids involved in α-SP MAb binding to SP.

MATERIAL AND METHODS

Preparation of α-SP MAb

α-SP MAb was prepared from the supernatant of NC1/34 rat-mouse heterohybridoma and purified as described previously.[18]

Preparation of SP-BSA conjugate

SP (15 mg, 1.2 mL) and bovine serum albumin (BSA) (40 mg, 2.0 mL) were dissolved separately in sodium bicarbonate buffer (0.1 M, pH = 9.2) and mixed. Glutaraldehyde (0.5%, 0.6 mL) was added to the mixture and stirred for 2 h at room temperature (22-22°C). Glycine (75 mg) was added to the mixture which was stirred for 1 h at room temperature. This mixture was dialysed (3 d, 3 changes) against distilled water. The SP-BSA conjugate was distributed into vials and stored (-20°C) until used.

Measurement of relative affinities by ELISA

The relative affinities of the α-SP MAb for SP and its fragments and selective NK-receptor antagonists were determined by inhibition ELISA.[18] Briefly, microtiter plates (Nunc-Immuno Plate, MaxiSorp™) were coated with 50 μL of 1: 100 dilution of SP-BSA conjugate (containing 15 mg SP, 40 mg BSA, 75 mg glycine and 6 mL glutaraldehyde 5%, total volume 10 mL) and incubated overnight at 4°C. After washing (3x) with PBS/Tween (0.05%) (automatic plate washer, Molecular Devices, Maxline Model 4845-02), plates were blocked with BSA (1%) in PBS for 1 h at 37°C. Then, varying concentrations of SP, SP fragments, or selective tachykinin receptor antagonists (0.04-1.00 μM, 25 μL) were added to the wells; a constant amount of α-SP MAb solution was added and the plates incubated (1 h, 37°C). After washing (3x), rabbit anti-rat IgG antibody/HRPO conjugate was added and the plates incubated (1 h, 37°C). After washing (5x), 2,2;-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) was added and OD was measured at 405 nm after 15 min (Molecular Devices, MaxLine microplate washer Model 4845-02, SoftMax® Software Version 2.02). All assays were performed twice in duplicate. Changes in OD in the presence of different concentrations of SP, SP fragments, or selective NK-receptor antagonists were compared with values obtained in the absence of these peptides. Wells containing only SP-BSA and α-SP MAb, only SP-BSA and SP, or SP fragments and only α-SP MAb and SP or SP fragments were among the controls used. The relative affinities of α-SP MAb for SP and SP fragments were estimated by the method of Van Heyningen et al.[22] Data were fitted to curves using non-linear regression and the concentration of added SP, SP fragments, or selective NK-receptor antagonist that reduced OD to 50% of maximum (IC50) was estimated.

Synthesis of peptides

A SPOTs (solid phase synthesis of peptides) schedule was generated (SPOTs software, version 2). Fmoc-amino acid chlorides were used for the solid phase synthesis. Cellulose membranes with free OH groups were used as the solid phase. All amino acids used were of L configuration. Fmoc-amino acids were dissolved in 1-methyl-2-pyrrolidine (110-180 mg mL^{-1}). A 0.9 μL aliquot of the first amino acid was dispensed on to each of the SPOTs (color change from blue to blue/green, green or yellow) and repeated after 15 min. After 15 min, the trough containing the SPOTs membrane was placed on rocking table and dimethylformamide (DMF) was added to the trough and rocked (2 min). This step was repeated twice. To block any unreacted amino acid, the SPOTs membrane in the trough was treated with acetic anhydride (0.8 mL) in DMF (20 mL) and rocked (15 min). After washing with DMF (3x), excess acetic anhydride was eliminated by adding 20% piperidine in DMF and rocking (5 min). After washing with DMF (3x), 1% bromophenol blue solution in DMF was added to SPOTs membrane and rocked (5 min). After washing with methanol (3x), the SPOTs membrane was wrapped in chromatography paper and dried in a cool air dryer. This procedure was repeated for each cycle in which an amino acid was added, except the last. For the last cycle the amino acid was added as above. After washing (3x) with DMF, 20% piperidine/DMF was added. Then, after washing (3x) with DMF, color was developed with bromophenol blue solution. After washing with DMF (3x), acetic anhydride was added. Then, after washing with DMF (3x) and with methanol (3x), the SPOTs membrane was dried.

After the last cycle, the side chain deprotection was performed by adding a mixture of dichloromethanol: trifluoroacetic acid: triisobutylsilane (1:1:0.05) to the SPOTs membrane in a propylene box and rocking (1 h). After washing with dichloromethane (3x) and methanol (3x), the SPOTs membrane was dried, placed in a plastic bag and stored (-20°C).

The sequence of each of the peptides synthesized using SPOTs is shown in Table I.

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Table I. Amino acid sequences of SP6-11 and SP analogues synthesized by SPOTs analysis. Double line represents the cellulose membrane.

SP^{6-11}						NH_2	Gln	Phe	Phe	Gly	Leu	Met	COOH
1						NH_2	Gln	Phe	Phe	Gly	Leu	***Ala***	COOH
2						NH_2	Gln	Phe	Phe	Gly	***Ala***	Met	COOH
3						NH_2	Gln	Phe	Phe	***Ala***	Leu	Met	COOH
4						NH_2	Gln	Phe	*Ala*	Gly	Leu	Met	COOH
5						NH_2	Gln	***Ala***	Phe	Gly	Leu	Met	COOH
6						NH_2	***Ala***	Phe	Phe	Gly	Leu	Met	COOH
7		NH_2	Gln	Phe	Phe	Gly	Leu	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
8		NH_2	Gln	Phe	Phe	Gly	Leu	***Ala***	**Arg**	**Arg**	**Arg**	**Arg**	COOH
9		NH_2	Gln	Phe	Phe	Gly	***Ala***	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
10		NH_2	Gln	Phe	Phe	***Ala***	Leu	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
11		NH_2	Gln	Phe	***Ala***	Gly	Leu	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
12		NH_2	Gln	***Ala***	Phe	Gly	Leu	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
13		NH_2	***Ala***	Phe	Phe	Gly	Leu	Met	**Arg**	**Arg**	**Arg**	**Arg**	COOH
SP	HOOC	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg	NH_2
14	NH_2	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg	COOH
15	NH_2	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	***Ala***	COOH
16	NH_2	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	***Ala***	Arg	COOH
17	NH_2	Met	Leu	Gly	Phe	Phe	Gln	Gln	Pro	***Ala***	Pro	Arg	COOH
18	NH_2	Met	Leu	Gly	Phe	Phe	Gln	Gln	***Ala***	Lys	Pro	Arg	COOH
19	NH_2	Met	Leu	Gly	Phe	Phe	Gln	***Ala***	Pro	Lys	Pro	Arg	COOH
20	NH_2	Met	Leu	Gly	Phe	Phe	***Ala***	Gln	Pro	Lys	Pro	Arg	COOH
21	NH_2	Met	Leu	Gly	Phe	***Ala***	Gln	Gln	Pro	Lys	Pro	Arg	COOH
22	NH_2	Met	Leu	Gly	***Ala***	Phe	Gln	Gln	Pro	Lys	Pro	Arg	COOH
23	NH_2	Met	Leu	***Ala***	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg	COOH
24	NH_2	Met	***Ala***	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg	COOH
25	NH_2	***Ala***	Leu	Gly	Phe	Phe	Gln	Gln	Pro	Lys	Pro	Arg	COOH

Direct ELISA on SPOTs membrane (dot immunoassay)

All steps were carried out at room temperature. The SPOTs membrane was allowed to warm to room temperature, rinsed with methanol and dried. After washing (3x) with PBS for 10 min, blocking buffer (BSA 3% in PBS) was added to the SPOTs membrane and it was rocked overnight. After washing (3x) with PBS, α-SP MAb or polyclonal anti-SP was added to the SPOTs membrane and it was rocked for 4 h. After washing (3x) with PBS, second antibody (rabbit anti-rat IgG/HRPO conjugate or goat anti-rabbit IgG/HRPO conjugate) was added to the SPOTs membrane and it was rocked for 2 h. After washing (3x) with PBS, substrate (TMB-Blotting™ or 4-chloro-1-naphthol) was added and the membrane rocked (10-60 min) to distinguish between positive and negative SPOTs.

Peptide regeneration

After immunoassay, the SPOTs membrane was washed (3x, 20 mL, 10 min) with MilliQ water, then with DMF (3x, 10 mL, 10 min). The membrane was washed (3 x, 20 mL, 10 min) with regeneration buffer A (urea 48% and sodium dedocyl sulphate 1% in MilliQ water) and then (3x, 20 mL, 10 min) with regeneration buffer B (MilliQ water: ethanol: acetic acid, 4: 5: 1). Finally, it was washed (3x, 20 mL, 10 min) with methanol, before drying and stored in a plastic bag at -20°C.

Materials

ABTS peroxidase substrate (Kirkegaard & Perry Labs., USA); substance P (Peptide Institute Inc., Japan); goat anti-rabbit IgG antibody/HRPO conjugate, rabbit anti-rat IgG antibody/HRPO conjugate, N,N-dimethylformamide, 1-methyl

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2-pyrrolidine, 4-chloro-1-naphthol, SP^{9-11}, SP^{8-11}, SP^{7-11}, SP^{6-11}, SP^{5-11}, SP^{4-11}, SP^{3-11}, SP^{2-11}, SP^{1-4}, SP^{1-7} and SP^{1-9} (Sigma, USA); rabbit anti-substance P antibody (ICN Biochemical Inc., USA); ethyl alcohol and methyl alcohol (Mallinckrodt Specialty Chemicals Canada Inc., Canada); acetic acid and acetic anhydride (Anhaler, BDH Inc. Canada); dichloromethane, piperidine and methylene chloride (Fisher Scientific Limited, Canada); bromophenol blue, trifluoroacetic acid and triisobuthylsilane (Aldrich Chemical Company, USA); Fmoc-amino acids, methionine, Phe-Phe, Phe-Gly, Gly-Leu and Leu-Met (Bachem Bioscience Inc., USA); sodium dodecyl sulphate and urea (Gibco BRL, USA); 3,3',5,5'-tetramethylbenzidine (TMB-BlottingTM, Pierce, USA). CP 96,345 was a gift of Pfizer Inc., Groton, CO; SR 48,968 (gift of Sanofi Récherche, Montpellier, France).

Statistical analyses

Data from ELISA were analyzed with SoftMax®, Microsoft Excel® and Mathematica® softwares. Inhibition curves were fitted using non-linear regression and compared via 95% confidence limits. IC50s, with 95% confidence limits, were calculated from the curves. The Mann-Whitney rank sum test and Kruskal-Wallis ANOVA on ranks were used to test the differences between the inhibition curves. Significance was assumed at the 5% level.

RESULTS

Relative affinities of α-SP MAb for SP, its fragments and selective NK-receptor antagonists

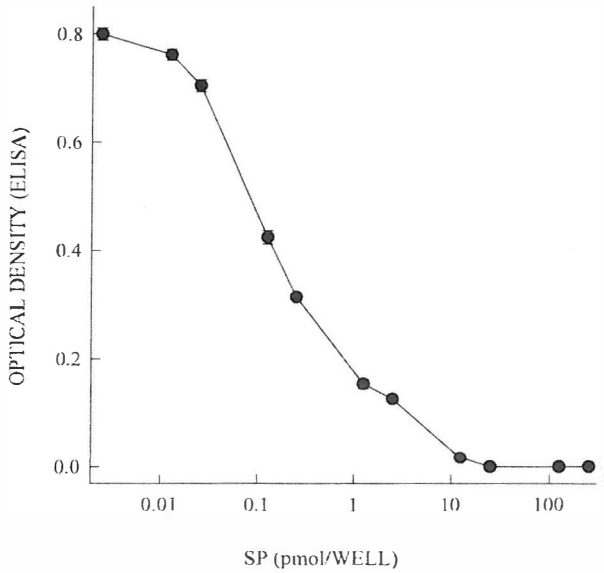

Fig. 1. Dilution curve for the α-SP MAb obtained using a constant, predetermined amount of SP-BSA conjugate. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

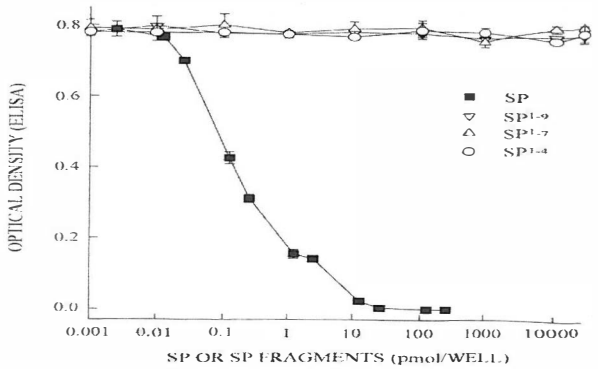

Fig. 2. Relative affinities of α-SP MAb for SP and its N-terminal fragments evaluated with a competitive binding technique in the presence of a constant amount of SP-BSA conjugate and α-SP MAb and different concentrations of SP, SP^{1-9}, SP^{1-7} or SP^{1-4}. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

Using ELISA, a dilution curve was established using a standardized amount of SP-BSA conjugate to capture different dilutions of α-SP MAb (Fig. 1). From this, it was determined that a 1:1000 dilution of α-SP MAb solution was suitable for the inhibition ELISA and 25 μL/well of this was used in all assays. Findings from the inhibition ELISA are summarized in Table II and Figs. 2-7. SP^{1-4}, SP^{1-7} and SP^{1-9}, methionine, the dipeptides, Phe-Phe, Phe-Gly, Gly-Leu and Leu-Met and the selective NK-receptor antagonists (NK1: CP 96,345; NK2: SR 48,968) did not alter α-SP MAb binding (Figs. 2-4). By contrast, SP and its C-terminal fragments containing >3 amino acids prevented α-SP MAb binding. The relative affinities of SP and its fragments were obtained from the inhibition curves. IC50s are summarized in Table II.

Interestingly, SP^{6-11} and SP^{7-11} were 250 and 40 times more potent than SP itself at preventing binding (Fig. 5), SP^{5-11}, SP^{4-11}, SP^{3-11} and SP^{2-11} were approximately as active as SP (Fig. 6) and SP^{8-11} or SP^{9-11} were less active than SP (Fig. 7).

Alanine scanning

The peptides prepared are summarized in Table I. The activities of these peptides were estimated by dot immunoassay using monoclonal α-SP MAb and α-SP serum. Colour was developed in dots with 4-chloro-1-naphthol as TMB-Blotting™ failed to yield any color. Using this reagent, none of the analogues showed any evidence of binding with monoclonal α-SP MAb; all showed evidence of binding to polyclonal α-SP serum.

DISCUSSION

The relative affinities of the α-SP MAb for SP and its various fragments were estimated by inhibition ELISA.

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Table II. Relative affinities of α-SP MAb for SP and SP fragments.

Amino acid sequence											Abbreviation	Relative affinity
1	2	3	4	5	6	7	8	9	10	11		
Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met $CONH_2$	SP	1
								Gly	Leu	Met $CONH_2$	SP^{9-11}	4×10^{-4}
							Phe	Gly	Leu	Met $CONH_2$	SP^{8-11}	7.8×10^{-3}
						Phe	Phe	Gly	Leu	Met $CONH_2$	SP^{7-11}	4.0×10^{1}
					Gln	Phe	Phe	Gly	Leu	Met $CONH_2$	SP^{6-11}	2.5×10^{2}
				Gln	Gln	Phe	Phe	Gly	Leu	Met $CONH_2$	SP^{5-11}	6.3×10^{-1}
			Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met $CONH_2$	SP^{4-11}	6.7×10^{-1}
		Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met $CONH_2$	SP^{3-11}	1.2
	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met $CONH_2$	SP^{2-11}	8.3×10^{-1}
Arg	Pro	Lys	Pro	$CONH_2$							SP^{1-4}	$<4.8\times10^{-6}$
Arg	Pro	Lys	Pro	Gln	Gln	Phe	$CONH_2$				SP^{1-7}	$<4.8\times10^{-6}$
Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	$CONH_2$		SP^{1-9}	$<4.8\times10^{-6}$

In this competitive binding technique, a constant amount of SP-BSA conjugate bound to the plate was used to capture α-SP MAb that had not bound to different concentrations of SP or its fragments in solution.[18] The amount of α-SP MAb added was determined from a dilution curve and kept constant in all experiments. This ensured reproducible measurements of relative affinity. The findings indicated that the α-SP MAb had approximately the same affinity for SP^{2-11}, SP^{3-11}, SP^{4-11} and SP^{5-11} as SP itself. By contrast, the α-SP MAb's affinity for SP^{6-11} and SP^{7-11} was respectively 250 and 40 times greater than for SP. However, α-SP MAb's affinity for SP^{8-11} and

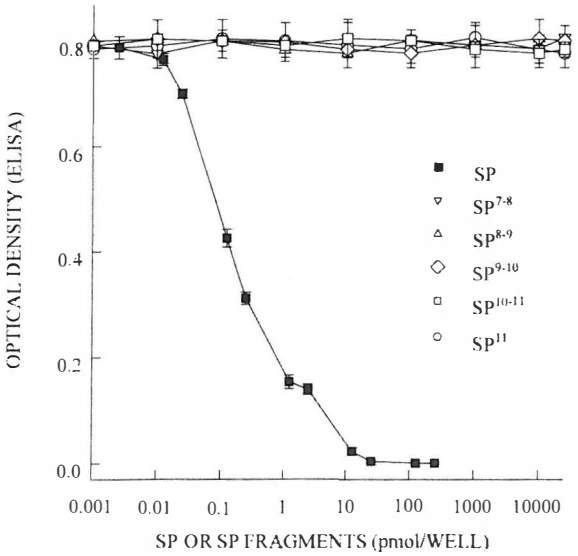

Fig. 3. Relative affinities of α-SP MAb for SP and mono- or di-peptide fragments of SP evaluated with a competitive binding technique in the presence of a constant amount of SP-BSA conjugate and α-SP MAb and different concentrations of SP, SP^{7-8}, SP^{8-9}, SP^{9-10}, SP^{10-11} or SP^{11}. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

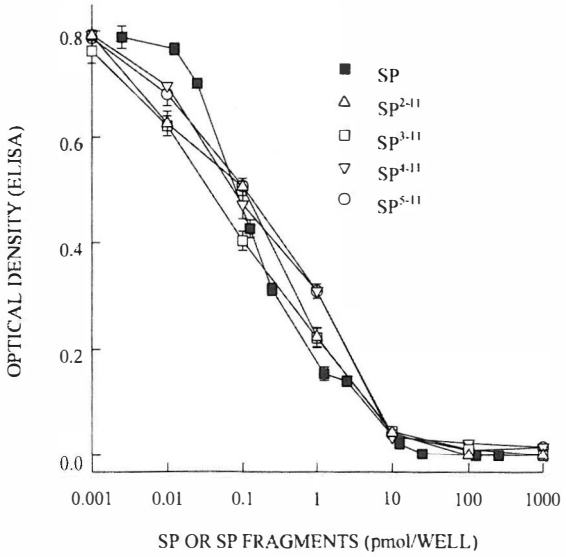

Fig. 4. Relative affinities of α-SP MAb for SP and its C-terminal fragments evaluated with a competitive binding technique in the presence of a constant amount of SP-BSA conjugate and α-SP MAb and different concentrations of SP, SP^{2-11}, SP^{3-11}, SP^{4-11} or SP^{5-11}. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

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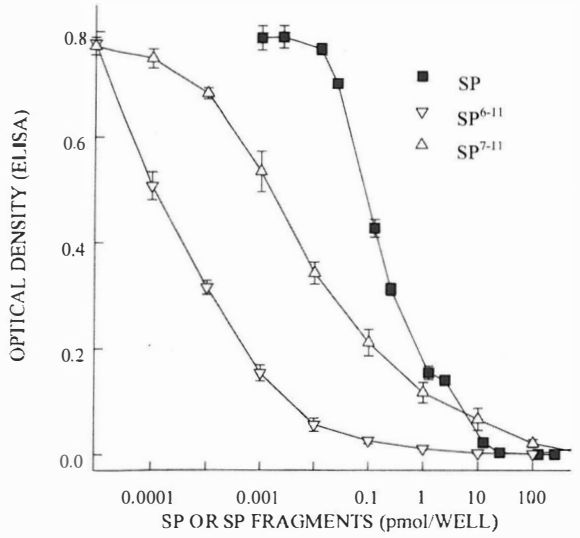

Fig. 5. Relative affinities of α-SP MAb for SP and its C-terminal fragments evaluated with a competitive binding technique in the presence of a constant amount of SP-BSA conjugate and α-SP MAb and different concentrations of SP or SP^{6-11} and SP^{7-11}. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

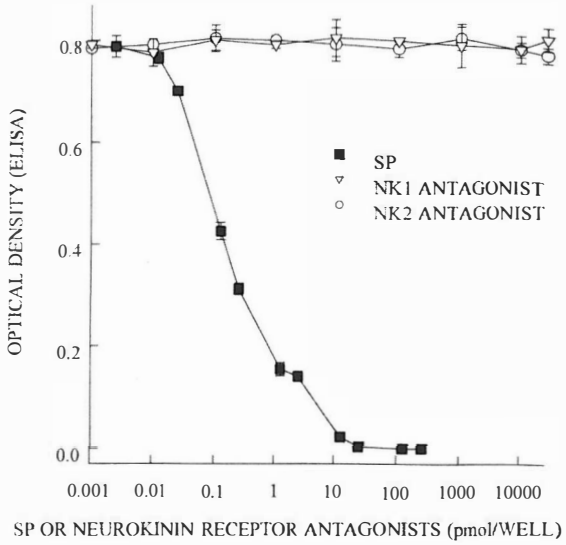

Fig. 7. Relative affinities of α-SP MAb for SP and selective neurokinin receptor antagonists evaluated with a competitive binding technique in the presence of a constant amount of SP-BSA conjugate and α-SP MAb and different concentrations of SP or selective neurokinin receptor antagonists. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

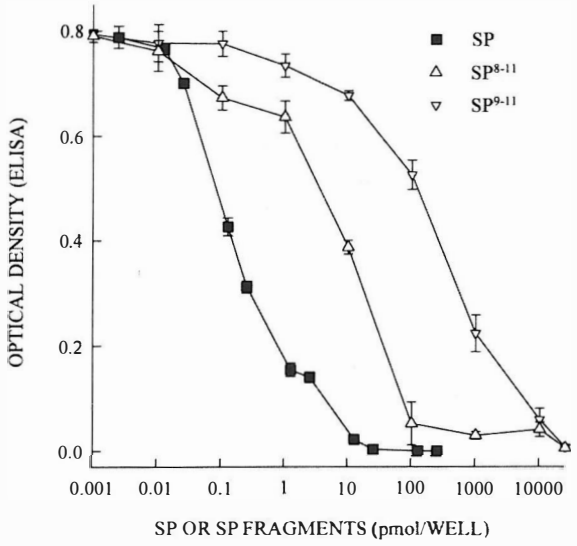

Fig. 6. Relative affinities of α-SP MAb for SP or its C-terminal fragments evaluated with a competitive binding technique in the presence of a constant amount of SP-BSA conjugate and α-SP MAb and different concentrations of SP, SP^{8-11} or SP^{9-11}. The amount of α-SP MAb captured by the SP-BSA conjugate is shown as optical density of ELISA.

SP^{9-11} was less than that for SP. In agreement with earlier findings,[17] the data indicated that α-SP MAb is directed against C-terminus of SP. Moreover, amino acids 6 and 7, and, to a lesser extent, amino acids 8 and 9, play a crucial role in determining the α-SP MAb's affinity for SP and its fragments. Interestingly, in this system, the α-SP MAb showed no affinity for N-terminal SP fragments and mono- or di-peptides derived from SP's C-terminal, confirming the crucial role of all the four amino acids in the C-terminal of SP in determining affinity. The NC1/34 was generated by immunizing mice with an antigen prepared by using glutaraldehyde to couple SP via its N-terminal to keyhole limpet hemocyanin. Thus, the α-SP MAb should be directed towards an epitopic site on the C-terminal part of the molecule. Similarity in the C-terminus of tachykinins could account for weak cross reactivity of α-SP MAb with eleidosin and NKA reported previously.[17-18] It is possible that conjugating SP to BSA and absorbing it onto the plate alters or impedes expression of its relevant epitopic sites. Thus, the conjugate's ability to compete for α-SP MAb with free SP or SP fragments is probably reduced. Thus, this is not a true "competitive" inhibition assay. However, as the conditions were similar for SP and SP fragments, it is likely that the values obtained for the α-SP MAb's relative affinities for them are representative. It was noteworthy that the α-SP MAb's affinity decreased from a peak at SP^{6-11} to values around that for SP for fragments SP^{5-11}, SP^{4-11},

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SP^{3-11} and SP^{2-11}. This difference may be related to the fragments' molecular size or changes in their conformation in solution. SP^{6-11} and NKA^{6-11} differ by two amino acids at positions 8 and 6 - Phe vs Val; Gln vs Ser, respectively. The commonality at amino acids 7 (Phe vs Phe) and 9 (Gly vs Gly) may contribute to the cross reactivity observed.

The use of monoclonal antibodies against tachykinins appears to be a powerful approach for detecting tachykinins and investigating their roles. This approach has been used by several investigators.[17, 19, 20-21] However, similarity in the C-terminus of tachykinins may preclude the use of monoclonal antibodies directed towards the C-terminus of tachykinins. Thus, detection of any activity using these antibodies could be referred to tachykinin-like immunore-activity. To detect and differentiate between the effects of tachykinin molecules, it is necessary to use highly specific monoclonal antibodies with minimal cross reactivity with other related peptides. Antibodies directed against the respective N-terminus of tachykinins, where there is considerable variability among tachykinins, look promising for this purpose.

De novo peptide synthesis was used to try and confirm the conclusions drawn from measurements of relative affinities by inhibition ELISA. There are two different ways to create a defined set of peptide sequences: the genetic and the synthetic approach.[23] The latter approach was adopted for these studies. It was decided to use the SPOTs solid phase synthesis system. With this method, numerous peptides can be synthesized simultaneously on a derivatized cellulose membrane. Peptide "SPOTs" can then be evaluated using a dot immunoassay. Analogues of SP^{6-11} were prepared with single sequential substitutions of alanine for each amino acid (Table I). As SPOTs syntheses utilized the N-terminal for the addition of amino acids to the peptide, similar peptides in which the C-terminal was "spaced" away from the membrane by four arginine molecules to expose the methionine residue were also prepared. Lastly, analogues of SP were prepared in which the C- and N-terminals were reversed. Dot immunoassay of all these peptides revealed that none of these peptides bound the monoclonal α-SP MAb, but all of them bound the polyclonal α-SP serum used as a positive control. These findings indicate the importance of an accessible and untreated C-terminal portion of the peptide for binding to the α-SP MAb. Addition of spacer arginine molecules clearly did not provide this. The SPOTs system used the C-terminal to bind the peptide to the membrane and added amino acids via the N-terminal. Since the C-terminal is an important contributor to the α-SP MAb's affinity for peptides, this method and alanine replacement studies unfortunately did not provide information about the role of specific amino acids in binding to α-SP MAb.

Lastly, we attempted to determine whether there were any similarities between the α-SP MAb's paratopic site(s) and NKR by measuring the Ab's affinity for the selective NK1-receptor antagonist CP 96, 345 (Snider et al., 1991) and the selective NK2-receptor antagonist SR 48, 968 (Emonds-Alt et al., 1992). NK1 and NK2 receptors belong to the family of G-protein-coupled receptors.[24-25] Neurokinin agonists and antagonists bind to the receptors' extracellular domains.[11,26] The structure-activity relation-ships involved in SP's binding have been much studied.[27-28] The smallest fragment with significant affinity for NKR is SP^{6-11}.[29-30] Interestingly, Wang et al. showed that changes of chirality of the amino acids in the C-terminal of SP markedly altered affinity for the NK1-receptor. By contrast, changes at the N-terminal had much less effect.[31] In our experiments, the α-SP MAb bound neither the NK1- nor the NK2-receptor antagonist suggesting that the paratopic site on the antibody bears little resemblance to the binding sites on NK-receptors.

In conclusion, these experiments show that the monoclonal α-SP MAb derived from the rat/mouse heterohybridoma NC1/34 is directed against epitopic sites on SP's C-terminal. Amino acids 6 and 7, and, to a lesser extent, 8 and 9, are important in determining affinity. The methionine residue in the C-terminal of SP and SP fragments has to be exposed for binding to occur.

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