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Measurement of 150 kDa protein of *Taenia solium* metacestodes by antibody-sandwich ELISA in cerebrospinal fluid of neurocysticercosis patients*

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Abstract: An antigenic protein in cystic fluid of *Taenia solium* metacestodes (CF) of 150 kDa was measured by antibody-sandwich ELISA in serum and cerebrospinal fluid (CSF) of neurocysticercosis patients. Capture antibodies were rabbit antisera against CF (RACF) and a monoclonal antibody (MAb) against 150 kDa protein in CF. Lower limit of antibody-sandwich ELISA was 8 ng/ml of the protein. Except CF, no tested helminthes extracts reacted. Levels of the protein in 351 sera from 255 patients (55 surgery confirmed and 202 antibody and CT/MRI confirmed) were below sensitivity of the assay. Of 276 CSF from 212 patients, 31 samples (11.2%) showed positive findings. This assay, therefore, was not sensitive enough to be a diagnostic. Instead, the 150 kDa protein appeared in CSF in such situations as in 2 days after praziquantel treatment, or as in a patient infected with a racemose cysticercus with degenerated cyst wall. Of cases whose follow-up CSF were assayed, 2 cases showed that the protein appeared intermittently. These results suggest strongly that appearance of free 150 kDa protein is associated with cyst wall rupture. In CSF which contained the 150 kDa protein over 61 ng/ml, the protein was recognized in SDS-PAGE before and after immunoprecipitation.

Key words: *Taenia soilum* metacestodes, neurocysticercosis, antigenemia, antibody-sandwich ELISA

INTRODUCTION

Of helminthic infections causing neurologic diseases, neurocysticercosis (NCC) is the most important in the world. For the proper management of NCC patients, correct differential diagnosis is a prerequisite. Definitive diagnosis of NCC is made when the metacestode was removed by surgery. Since the 1980s, however, non-surgical approaches to diagnosis and treatment become clinical modes, because the imaging diagnosis is available together with effective anthemintics (Nash and Neva, 1984). Antibody test for NCC, therefore, became an important complementary tool of diagnosis because CT/MRI findings in NCC patients are extremely protean according to the stages, location and number of the metacestode infection. Many NCC patients reveal ambiguous imaging findings to diagnose indeed (Chang *et*

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al., 1991).

Specific antibody test by ELISA or by enzyme-linked immunoelectrotransfer blot (EITB) became standard techniques of the immunodiagnosis (Cho *et al.*, 1986; Tsang *et al.*, 1989). One of main problems in the antibody tests is their sensitivity especially in cases infected with one or a few worms or with calcified parasites (Chang *et al.*, 1988; Wilson *et al.*, 1991). To improve the sensitivity of the serologic diagnosis of NCC, antigen detection in CSF has been tried (Estrada and Kuhn, 1985; Tellez-Giron *et al.*, 1987; Correa *et al.*, 1989; Choromanski *et al.*, 1990). In their reports, capture ELISA for antigen detection exhibited lower sensitivities than antibody tests.

In this study, we tried to measure levels of a specific antigen of 150 kDa protein in sera and CSF from NCC patients. In addition to an evaluation of the antibody-sandwich ELISA as a diagnostic tool, the appearance of the protein in CSF was analysed in different situations of neurocysticercosis patients.

MATERIALS AND METHODS

1. Antibody-sandwich ELISA

(1) **Subjects to the test:** As shown in Table 1, a total of 629 samples from 264 NCC cases was tested by antibody-sandwich ELISA. The samples consisted of 351 sera, 276 CSF and 2 cystic fluid. The cystic fluid were surgically collected from brain lesion. In 55 patients, diagnosis of NCC was made by neurosurgery or by biopsy of subcutaneous nodule. In the remaining 202 patients, the diagnosis was made by positive specific antibody test either in serum or in CSF or in both by ELISA (Cho *et al.*, 1986) together with compatible findings in brain CT/MRI. In surgically diagnosed cases, antibody levels in serum or CSF may be in negative range because patients with a single low densities were confirmed by neurosurgery. In cases diagnosed by antibody test, antibody levels were all in positive range. The test samples of NCC were randomly selected out of our Department pool of the parasite antibody screening system for neurologic diseases.

(2) **Capture antibodies:** RACF was prepared by immunizing rabbits with CF. Immunizing procedure was described in Kong *et al.* (1992). Briefly, rabbits were injected subcutaneously with 100 μg of CF, mixed in same volume of Freund's complete adjuvant. CF in Freund's incomplete adjuvant was boosted twice in 2 weeks interval. Four days before bleeding, 10 μg of CF was injected intravenously. RACF was secured by heart puncture. Antibody level in RACF was measured by ELISA using peroxidase-conjugated anti-rabbit IgG (light- and heavy-chain specific, Cappel, U.S.A.).

MAb against 150 kDa protein in CF (McAb CF298.18), prepared by Kim *et al.* (1986), was used in this study. This MAb was bound to the major protein component of CF, a 150 kDa (band C protein in non-denaturing disc-PAGE, Kim *et al.*, 1986). When observed by reducing or non-reducing SDS-PAGE, the 150 kDa

Table 1. Number of NCC cases and their samples assayed in this study

Category	Number of cases	No. positive/No. of samples		
		serum	CSF	cystic fluid
Serum only tested	50	0/60	(−)*	(−)
CSF only tested	7	(−)	0/7	(−)
Serum/CSF tested once	162	0/183	15/162	(−)
Serum/CSF tested repeatedly	43	0/108	16/107	(−)
Cystic fluid tested	2	(−)	(−)	2/2
Total	264	0/351	31/276	2/2

(−): not done

protein cleaved into subunits of 15, 10 and 7 kDa (Cho *et al.*, 1988).

(3) **Procedures of the antibody-sandwich ELISA:** After chequerboard titration of RACF, CF, MAb, peroxidase-conjugated antimouse IgG (heavy- and light-chain specific, Cappel, U.S.A.), the following conditions of ELISA was set.

RACF was diluted 1 : 100,000 in carbonate buffer (pH 9.6) and 200 μl was allocated to each well of polystyrene microtiter plate (Costar, U.S.A.) at 4°C overnight. The wells were washed with phosphate buffered saline/0.05% Tween 20 (PBS/T, pH 7.3) 3 times. Two hundred μl of samples (undiluted sera/CSF or other samples) were reacted at 37°C for 2 hours. The wells were washed 3 times again with PBS/T. 1 : 5,000 diluted MAb in PBS/T was reacted at 37°C for 2 hours again. After washing, peroxidase-conjugated anti-mouse IgG diluted 1 : 1,000 in PBS/T was reacted for 2 hours. After final washing, substrate, consisting of 99 ml of distilled water, 1 ml of 1% *o*-phenylene diamine and 10 μl of 30% H_2O_2, was reacted for 30 minutes. The coloring reaction was stopped by adding 25 μl of 8 N H_2SO_4. Absorbance (abs.) was read at 490 nm using Microplate Reader (Model 3,550, Bio-Rad, U.S.A.).

In each test of ELISA, 2-fold diluted CF, from 1 : 500 to 1 : 1,024,000 in PBS/T was tested along with samples to make a standard curve for the concentration of CF protein. PBS/T was tested as a negative control in each test.

(4) **Other parasite antigens:** To observe any non-specific cross reaction, antibody-sandwich ELISA was undertaken with other parasite antigens. The tested antigens were: crude saline extract of adult *Paragonimus westermani*, adult *Clonorchis sinensis*, adult *Metagonimus yokogawai*, adult *Fasciola* species, larval *Spirometra mansoni* plerocercoid (sparganum), larval *Anisakis* and muscle larva of *Trichinella spiralis*. The protein contents in the antigen samples were 2.2~6.5 mg/ml as measured by Lowry *et al.* (1951). These antigens were tested in dilution of 1 : 500 in PBS/T.

2. **SDS/PAGE and Immunoprecipitation**

(1) **Reducing SDS-PAGE:** To demonstrate the subunits of the 150 kDa protein in the antigen positive samples of neurocysticercosis, SDS-PAGE of reducing condition of Laemmli (1970) was done. Procedures of SDS-PAGE was described in Cho *et al.* (1988).

(2) **Immunoprecipitation:** Patient samples of CSF and cystic fluid, which were proved as 150 kDa protein positive by antibody-sandwich ELISA were tested again by immunoprecipitation (Harlow and Lane, 1988) to confirm presence of the protein. Briefly, 50 μl of CSF or cystic fluid from the patients were mixed with 10 μl Pansorbin (Calbiochem, U.S.A.). Non-specific IgG was precleared at 4°C. Supernatant was obtained by centrifugation at 13,000 *g* for 5 minutes. 10 μl of McAb CF298.18 was added and incubated at 4°C for an hour. The immune complex was precipitated with 20 μl of Pansorbin at 4°C for an hour. The immunoprecipitate was washed with PBS/T 5 times prior to SDS-PAGE. Electrophoresis was carried out in 10~17.5% separating gel in reducing condition at 30mA constant current. The gel was stained with Coommassie blue R-250 or immunoblotted with RACF.

RESULTS

1. Standard curve of the antibody-sandwich ELISA

As shown in Fig. 1, relation between the 150 kDa protein concentration in CF and abs. by antibody-sandwich ELISA was linear in protein range of 8~60 ng/ml. The concentration of the 150 kDa protein was calculated as 70% of whole proteins in CF (6.0 mg/ml) as measured by densitometry (Cho *et al.*, 1988). By the antibody-sandwich ELISA, abs. was fluctuated below 0.05~0.10 when CF was diluted 1 : 512,000 or more (Fig. 1). Therefore, lower sensitivity limit of the ELISA was determined to be 8.2 ng/ml of 150 kDa protein in CF. When concentration of the 150 kDa protein is over

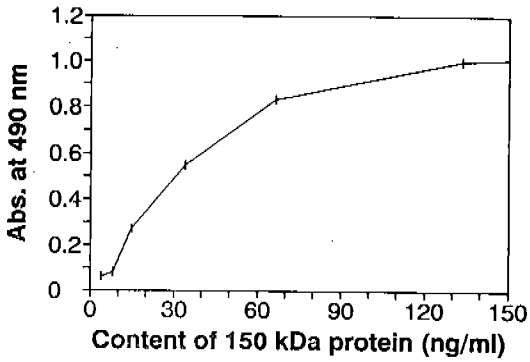

Fig. 1. Relation between 150 kDa protein concentration in CF and absorbance by antibody-sandwich ELISA. The concentration was calculated as 70% of whole proteins in CF (6.0 mg/ml) as measured by densitometry (Cho *et al.*, 1988). The standard curve was linear in protein range of 8~60 ng/ml. This range was considered to be the best measurement interval for the 150 kDa protein.

61 ng/ml, abs. were making a plateau. The range of 8~60 ng/ml was considered to be the best measurement interval.

2. Specificity of antibody-sandwich ELISA

The specificity of measuring 150 kDa protein by antibody-sandwich ELISA was observed by testing 1 : 500 diluted crude extracts of *P. westermani*, *C. sinensis*, *M. yokogawai*, *Fasciola* sp., sparganum, *Anisakis* and *Trichinella*. All these antigens showed abs. ranges from 0.01 to 0.05, which are far below the sensitivity limit of the assay.

3. Measurement of the 150 kDa protein in patients

(1) Results in sera of NCC patients: As shown in Table 1, a total of 351 sera samples from 255 NCC patients was assayed. None of these sera showed the positive results above sensitivity of the assay. Repeated examinations of sera in the same patients after praziquantel treatment did not exhibit any positive result.

(2) Results in CSF of NCC patients: A total of 276 CSF was assayed. Of them, 31 samples (11.2%) revealed positive tests (Table 1). When counted in patient numbers, 27 cases (12.7%) of NCC showed once or more positive results out of 212 tested patients (Table 2). In 107 repeatedly examined CSF samples from 43 cases, positive results of the 150 kDa protein was obtained in 12 patients. Of them, 10 cases showed the positive result once either before or after praziquantel treatment. Only in 2 cases, the positive results were observed more than 2 times. The ranges of 150 kDa protein amount in CSF were shown in Table 2.

Table 2. Range of 150 kDa protein concentration (ng/ml) in CSF of NCC patients

	No. of cases(samples) tested		
	once	twice	thrice or more
Number of tested cases	169	28	15
Number of tested samples	169	56	51
Below the sensitivity	154	24(52)	7(39)
Positive results	15	4(4)	8(12)
Range of 150 kDa protein concentration			
8~20 ng/ml	10	3(3)	5*(5)
21~40 ng/ml	1	1(1)	0*(2)
41~60 ng/ml	0	0	1*(2)
61 ng/ml or over	4	0	2*(3)

* Case number was counted in higher concentration category in repeatedly positive cases.

(3) Findings in 3 specific NCC patients: The patient samples, tested in this study, were collected for diagnosis of NCC. In many occasions, the samples were tested again for evaluation or to confirm the serologic test results after praziquantel treatment. The interval between praziquantel treatment and follow-up test was 2 weeks or longer in all but a case. The NCC patient repeated antibody test 2 days after initiation of praziquantel. The results of specific (IgG) antibody tests and levels of 150 kDa protein were shown in Table 3. The 150 kDa protein appeared only in a CSF sample collected on 2 days after the treatment.

In another patient of NCC with headache, general weakness and ataxia due to hydrocephalus, diagnosis was made by the antibody test by ELISA. Praziquantel treatment was done for 2 weeks in 2 courses each in 3 months interval. Because severe headache persisted, surgery was done to remove racemose cysticercus from the

Table 3. Antibody and 150 kDa protein levels in serum and CSF of a NCC patient who was treated with praziquantel since Nov. 11, 1987

	Antibody level (abs.) in		150 kDa level (ng/ml) in	
	serum	CSF	serum	CSF
Nov. 9, 1987	0.84	0.84	(−)**	(−)
Nov. 13, 1987	1.07	0.81	(−)	49
Nov. 27, 1987	1.04	0.84	(−)	(−)
Dec. 29, 1987	0.75	0.68	(−)	(−)

*: Antibody levels were measured by ELISA (Cho *et al.*, 1986); cut-off abs. is 0.18 in both serum and CSF

**(−): Below sensitivity of antibody-sandwich ELISA

Table 4. Antibody and 150 kDa protein levels in serum and CSF of a NCC patient with hydrocephalus

	Antibody level (abs.) in		150 kDa level (ng/ml) in	
	serum	CSF	serum	CSF
Apr. 13, 1987	0.40	0.69	(−)	not done
Jun. 25, 1987*	0.97	0.92	(−)	(−)
Sep. 9, 1987	0.66	0.57	(−)	over 61
Sep. 21, 1987*	0.63	0.70	(−)	(−)
Jan. 5, 1988**	0.39	0.65	(−)	(−)
Jan. 18, 1988	0.24	0.29	(−)	55

*: Praziquantel treatment finished

**: Worm removal surgery was done

4th ventricle. The worm disclosed patchy degeneration of cyst wall histologically. The antibody levels and the 150 kDa protein levels of this patient were shown in Table 4. CSF levels of 150 kDa protein was recognized 2 times intermittently about 3 months after praziquantel treatment and after the worm removal.

In another patient of NCC whose brain CT showed multiple low densities around parasellar area and hydrocephalus, serum and CSF samples were examined 5 times in a year and 3 months period (Table 5). Antibody levels were always very high in both samples. The 150 kDa protein was detected in CSF in 4 of 5 assays with fluctuations in their levels. In the final examination after ventriculoperitoneal shunt operation, the protein level in CSF was very high.

Table 5. Antibody and 150 kDa protein levels in serum and CSF of another NCC patient with hydrocephalus and multiple low densities

	Antibody level (abs.) in		150 kDa level (ng/ml) in	
	serum	CSF	serum	CSF
Jul. 5, 1988	0.79	0.84	(−)	over 61
Oct. 14, 1988*	0.53	0.75	(−)	24
Jan. 25, 1989	0.65	0.95	(−)	38
Apr. 26, 1989	0.75	0.55	(−)	(−)
Oct. 24, 1989**	0.72	1.06	(−)	over 61

*: Praziquantel treatment finished

**: Ventriculoperitoneal shunt operation done

(4) 150 kDa protein in cystic fluid collected from brain lesions: In 2 patients of NCC, cystic fluid was collected by stereotaxic surgery. Surgeons referred them to us to confirm the fluid as a parasite origin. The contents of 150 kDa protein in the samples were all over 61 ng/ml.

4. Findings of SDS-PAGE and immunoprecipitation in CSF and cystic fluid samples positive for 150kDa protein

Fig. 2 shows reducing SDS-PAGE findings of CSF and surgically collected cystic fluid of

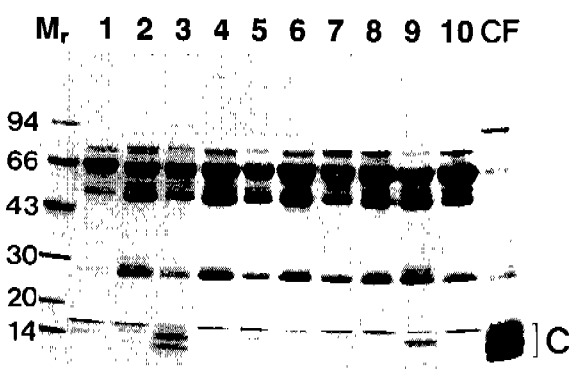

Fig. 2. SDS-PAGE findings of CSF and surgically collected cystic fluid of NCC patients. The samples were electrophoresed on 10~15% reducing gel with the supply of 30 mA constant current. M_r: Molecular mass in kDa, Lanes 1 and 5: Surgically collected cystic fluid, Lanes 2, 3, 4, 6, 7, 8, 9 and 10: NCC patients CSF, CF: Cystic fluid collected from a pig, C: Band C protein (150 kDa protein).

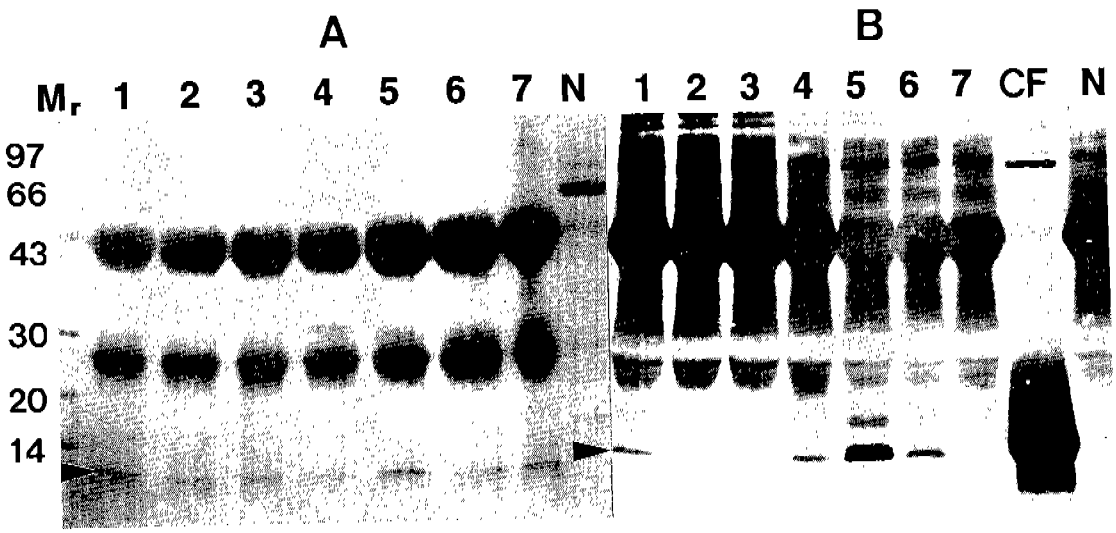

Fig. 3. SDS-PAGE and immunoblot findings of CSF from NCC patients after the immunoprecipitation. The proteins were separated using 10~17.5% gradient gel. M_r: Molecular mass in kDa, N: CSF from other neurologic patient, 1~7: CSF collected each NCC patient, CF: Cystic fluid collected from a pig. (A): MAb bound antigenic subunits of molecular mass of 10 kDa were recognized at the bottom of each lane (arrow head). (B): When this immune complex was immunoblotted with RACF, 10 kDa bands of 150 kDa protein were reacted (arrow head). Heavy and light chains of rabbit IgG (in Pansorbin) were non-specifically reacted with RACF.

NCC patients which contained 150 kDa protein over 61 ng/ml as measured by antibody-sandwich ELISA. In all samples, subunits of 150 kDa protein, *i.e.*, 15, 10 and 7 kDa were recognized at the bottom of each lane.

Fig. 3A show SDS-PAGE findings of the CSF and cystic fluid when observed after immunoprecipitation. MAb bound antigenic subunit of molecular mass of 10 kDa was recognized at the bottom of each lane. In Fig. 3B, the subunit of 150 kDa protein, 10 kDa band was recognized again at the bottom of each lane.

DISCUSSION

To investigate a possibility of higher sensitivity in antigen detection trials in NCC patients, excretory-secretory product or parenchymal antigens of *Taenia solium* have been tested by capture ELISA (Estrada and Kuhn, 1985; Tellez-Giron *et al.*, 1987; Correa *et al.*, 1989). The parasite antigen was detected in 10~70% of CSF samples of confirmed NCC patients when a polyclonal antibody toward metacestode antigen was used as a capture antibody (Estrada and Kuhn, 1985; Tellez-Giron *et al.*, 1987; Correa *et el.*, 1989) or when MAb were used (Correa *et al.*, 1989). In *Taenia saginata* cysticercosis in cattle, capture antibody ELISA using a MAb was reported to be capable of detecting the metacestode antigen in sera of cattle which are infected with more than 88 metacestodes (Brandt *et al.*, 1992). These results indicated that the antigen detection in sera or CSF of NCC patients are not sensitive as the specific-antibody (IgG) tests, unlike the expectations. Antibody tests in NCC, either by ELISA or by EITB, exhibit about 90% sensitivity when both serum and CSF was tested. Antibody test shows low sensitivity especially in patients with a single lesion and in those with calcified lesions (Chang *et al.*, 1988; Wilson *et al.*, 1991). In spite of this shortcomings of the antibody test, antigen detection by ELISA did not overcome the sensitivity problems. In this study, the antigen detection by antibody-sandwich ELISA was also not sensitive to be a diagnostic.

Choromanski *et al.* (1990) examined the presence and nature of parasite antigens in CSF of NCC patients. When patients CSF were filtered through HPLC, 6 protein peaks were separated. Of them, a protein of molecular mass of 110 kDa was the most antigenic when tested by ELISA using patients CSF. Nine of 23 CSF from confirmed NCC patients were positive for the 110 kDa protein. Though not certain and explicit at present, this 110 kDa protein of Choromanski *et al.* (1990) seems to be identical with the 150kDa proteins detected in this study if the protein is derived from CF of metacestodes. As confirmed by Cho *et al.* (1988) and Choi *et al.* (1990), main component proteins in CF were 150 kDa and 64 kDa. Of them, 150 kDa protein was separated into 3 subunits of 15, 10 and 7 kDa in reducing or non-reducing SDS-PAGE. These subunits were reported to be most antigenic in NCC patients when observed by immunoblot (Cho *et al.*, 1987).

In this study, presence of only the antigenic protein of 150 kDa in CF was assayed by antibody-sandwich ELISA. Our assay system was specific for the 150 kDa protein as demonstrated in the positive CSF by SDS-PAGE before and after immunoprecipitation using the MAb reacting to the protein. In SDS-PAGE, 3 subunits were clearly exhibited. This assay was specific too because helminth antigens so far screened exhibited negative results. In any serum sample, the 150 kDa protein was not detected probably because we did not separate antigen from immune complex. In 11.2% of tested CSF, however, the free 150 kDa protein was present in detectable range and sometimes even in high levels.

Considering the present results of antibody-sandwich ELISA, appearance of free antigenic protein in CSF seems to be related with cyst wall degeneration of *T. solium* metacestodes. Either by drug treatment or in natural degeneration of aged metacestodes, cyst wall rupture of metacestodes releases excess antigenic CF proteins including the 150 kDa into CSF. This temporary happening of the excess antigen in CSF seems to be managed by temporary inflammation and production of antibody in CSF in a few days. As shown in Table 3, a NCC patient treated by praziquantel showed free 150 kDa protein in 2 days after the treatment. In 26 patients tested about 14 days after praziquantel treatment in this study, 2 cases revealed free 150 kDa protein in CSF. These data are corresponding with the patients illness of headache and intracranial hypertension induced by praziquantel which are frequently observed in 3~10 days after the treatment. In addition, during the long, natural courses of cerebral cysticercosis, intermittent headache is frequent episodes in many patients. Results of this study raised the necessity of studies on the relations between CF protein release into CSF and resulting intracranial inflammatory processes.

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=국문초록=

뇌 유구낭미충증 환자 혈청 및 뇌척수액에서의 유구낭미충 낭액항원의 측정

중앙대학교 의과학연구소 및 의과대학 기생충학교실
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뇌 유구낭미충증 진단에는 영상진단을 가장 널리 이용하고 있으나 낭미충증의 영상진단은 감염충체수, 감염경과 기간, 감염위치 및 포도낭미충 존재여부 등에 따라 소견이 매우 다양하므로 감별진단에 도움이 되는 혈청학적 진단이 요구된다. 이때에 이용하는 혈청학적 진단 방법은 특이항체를 혈청이나 뇌척수액에서 증명하는 것이나 몇 가지 경우에 위음성 반응을 나타내어 민감도가 낮은 결점이 있다. 민감도가 낮은 경우를 해결하는 방법으로서, 또 기생충에서 유래하는 특이항원을 측정함으로써 항체측정 때 나타나는 교차반응을 최소화하고, 충체검출에 준하는 진단방법으로서 유구낭미충 특이항원을 환자 혈청 및 뇌척수액에서 측정하는 연구를 시도하고 있다. 이 연구에서는 유구낭미충증 환자에서 이중항체-효소면역측정법을 실시하여 유구낭미충의 낭액 항원의 구성단백질을 측정하였다. 항원반응용 항체로는 낭액으로 감작시킨 토끼혈청과, 낭액을 구성하는 단백질중 150 kDa에만 반응하는 단클론항체를 이용하였다. 이중항체-효소면역측정법으로는 150 kDa 단백질을 8 ng/ml까지 측정할 수 있었고, 61 ng/ml 이상에서는 농도에 따른 흡광도 차이가 적었다. 이 방법으로 스파르가눔, 간흡충, 폐흡충, 요꼬가와흡충, 간질, 고래회충 제 3 기유충 및 선모충 등의 생리식염수 추출액을 검색한 바 모두 음성반응이어서 특이도가 높다고 판단하였다. 뇌 유구낭미충증으로 진단한 환자 255명의 혈청 351개를 측정한 바 측정 가능범위에 미치지 못하여 모두 음성이었다. 환자 212명에서 얻은 뇌척수액 276개 중 31개(11.2%)는 양성이었다. 이와 같이 민감도가 낮아 항원측정법은 진단용으로 이용하기 어렵다고 생각하였다. 뇌척수액에서 150 kDa 단백질이 증명된 환자는 프라지콴텔 치료 후 2일이 경과한 경우, 그리고 약제치료에도 불구하고 임상경과가 불량하여 결국 외과적으로 충체를 제거한 바 낭벽이 초점성으로 변성된 포도낭미충에 감염되었던 환자의 뇌척수액에서 불규칙하게, 양적으로는 일정하지 않게 출현하였다. 150 kDa 단백질은 뇌 낭종성 병변에서 채취한 낭미충 낭액에서도 측정한 수 있었다. 그리고 150 kDa 단백질의 농도가 61 ng/ml 이상인 뇌척수액이나 외과적으로 얻은 낭액으로 SDS-폴리아크릴아미드 전기영동을 실시하여 150 kDa 단백질의 subunit인 15, 10, 7 kDa를 증명할 수 있었다.

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