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The production and characterization of anti-*Naegleria fowleri* monoclonal antibodies

Jae-Sook Ryu and Kyung-il Im*

Department of Parasitology, College of Medicine, Hanyang University, Seoul 133-791 and Department of Parasitology, College of Medicine, Yonsei University, Seoul 120-752, Korea*

Abstract: *Naegleria fowleri*, a free-living amoeba commonly found in moist soil and fresh water, enters the body via the nasal mucosa and migrates along the olfactory nerve to the brain, where it causes acute amoebic meningoencephalitis. In the present study 7 clones secreting monoclonal antibodies(McAbs) against *N. fowleri* were produced and the effector function of them was investigated. Their isotypes were IgG_1 (Nf 1, Nf 154), IgG_3 (Nf 137) and IgA (Nf 1, Nf 2, Nf 256, Nf 279). Five McAbs (McAb Nf 2, Nf 279, Nf 27, Nf 154, Nf 137) were specific for *N. fowleri* by ELISA and recognized the antigenic determinants located on the trophozoite surface by IFAT and immunoperoxidase stain. These five McAbs had capacity to agglutinate *N. fowleri* trophozoites and inhibited the growth of the amoeba in culture medium. McAb Nf 2 inhibited proliferation of trophozoites *in vitro* significantly. Also the cytotoxicity of *N. fowleri* against CHO cell was reduced in the presence of McAb Nf 2 and McAb Nf 154. From these results McAb Nf 2 was confirmed to weaken the virulence of the amoeba among 7 screened McAbs.

Key words: *Naegleria fowleri*, mouse, monoclonal antibody (McAb), surface membrane reactive McAb, protective effect of McAb

INTRODUCTION

Naegleria fowleri, a free-living amoeba commonly found in moist soil and fresh water, causes primary amoebic meningoencephalitis(PAM). About 140 cases of PAM have been reported worldwidely (Lallinger *et al.*, 1987). In Korea, human infection of PAM to date has not yet been proved, but only one probable *Acanthamoeba* meningoencephalitis case was informed in a Korean child (Ringsted *et al.*, 1976).

Because of rapid progress of the disease, immediate diagnosis and treatment are required. The diagnosis of this protozoan infection has been accomplished upon detecting the causative amoeba in CSF. Its fatality rate is greater than 95% with only 3 well documented survivors to date (Seidel *et al.*, 1982). Some understanding of effective treatment of this amoebic infection is still lacking.

In the present study, we prepared monoclonal antibodies (McAbs) specific for *N. fowleri* using hybridoma technique (Köhler and Milstein, 1975) and investigated the protective effect of McAb *in vitro*.

MATERIALS AND METHODS

I. Production of McAbs

1) Amoebae and culture: Amoebae used in this study were pathogenic *N. fowleri* ITMAP

359 (donated from Dr J.B. Jadin, Belgium), weakly pathogenic *N. jadini* 0400 and nonpathogenic *N. gruberi* EGB. In addition to these *Naegleria* spp. employed in this study, 2 *Acanthamoeba* spp. were used, pathogenic *A. culbertsoni* and nonpathogenic *A. royreba* OR strain. *Naegleria* spp. were grown axenically in a CGVS medium (Willaert, 1971) and *Acanthamoeba* spp. were cultured in CGV medium (Willaert, 1975).

2) **Immunization of BALB/c mice:** BALB/c mice were used in all experiments. Primary immunization consisted of subcutaneous injection of 6×10^6 formalin fixed *N. fowleri*, mixed with an equal volume of incomplete Freund adjuvant. After 2 weeks, mice were boostered twice intraperitoneally with amoebae, and followed by intravenous injection of amoebae 3 days before fusion.

3) **Fusion:** The spleen cells from immunized mice mixed with SP 2/0-Ag 14 myeloma cells and pelleted by centrifugation, and fused by resuspending the pellet in RPMI 1640 medium containing 50% (W/V) polyethyleneglycol (M. W. 4,000, Merck, Denmark). The cells were distributed in 100 μl in individual wells of 96 well flat-bottomed culture plate and were maintained at 37°C in 5% CO_2 incubator. One day after fusion, 100 μl of RPMI 1640 medium containing hypoxanthine (13.6 μg/ml) aminopterine (0.174 μg/ml) and thymidine (3.87 μg/ml) (HAT medium) were added to each well (Littlefield, 1964). HAT medium was changed at the third and fifth days after fusion, and hybrid cells were checked with an inverted microscope.

4) **Antibody assays of culture supernatants:** To select antibody-secreting hybrid cells out of grown colonies, the culture media in each well were screened by ELISA. Sonicated suspension of whole *N. fowleri* trophozoite antigen was pipetted into each well of 96-well plate. Each well was then incubated with culture supernatant. Peroxidase conjugated goat antimouse immunoglobulin was added, and bounded enzyme was detected by the addition of substrate. The reaction product was detected by an ELISA reader at 492 nm.

5) **Cell cloning:** Cloning was performed by limiting dilution by plating 0.3 cells into the individual wells (Mckearn, 1980). Normal BALB/c peritoneal exudate cells were used as a feeder layer. The culture media were screened for the presence of the antibody by ELISA.

6) **Mass production of McAb:** 0.5 ml of pristane (2, 6, 10, 14-tetramethyl pentadecane, Sigma, USA) was injected into BALB/c mice intraperitoneally and $5\times10^6\sim10\times10^6$ antibody-producing hybridoma cells were injected into the peritoneal cavity 1 to 3 weeks later. Ascitic fluid was obtained from peritoneal cavities 5 to 10 days later.

II. Characteristics of McAbs

1) **Antibody isotyping:** The isotype of each McAb was determined by a sandwitch ELISA using rabbit antimouse subclass specific Igs and a peroxidase conjugated goat anti-rabbit serum (Hyclone Lab, Logan, Utah).

2) **Immunological specificity of anti-*Naegleria fowleri* McAbs:** To screen the reactivity of 7 McAbs with other free-living amoebae than *N. fowleri*, ELISA was done. Other *Naegleria* spp. were used as a source of antigens, *N. fowleri* ITMAP 359, *N. gruberi* EGB, and *N. jadini* 0400. In addition to these *Naegleria* spp., *Acanthamoeba culbertsoni* and *A. royreba* OR were also used.

3) **Indirect fluorescent antibody test (IFAT) and immunoperoxidase stain:** An IFAT on live organisms were performed to determine whether McAbs reacted to the antigens on the surface membrane of the parasites (Ferrante and Thong, 1979). *N. fowleri* trophozoites were resuspended in culture supernatant containing McAb and incubated for 30 mins at 4°C. Afterwards, amoebae were resuspended in rabbit antimouse immunoglobulin labeled with fluorescein isothiocyanate (Cappel, Pennsylvania) at 1 : 30 dilution and incubated for 30 mins at 4°C. Amoebae were fixed in 4% formaldehyde for 2 hrs and were washed.

Amoebae were counterstained with 0.1% Evans blue for 5 mins and were allowed to air dry on a glass slide and coverglass was mounted.

For immunoperoxidase stain, *N. fowleri* trophozoites were fixed in 4% paraformaldehyde and incubated with culture supernatant containing McAb Nf 154 and reacted with peroxidase conjugated goat antimouse immunoglobulin. Then the sediment were fixed in 3% glutaraldehyde and hardened with 2% agar solution, and the block was cut in 1 mm thickness. The specimens were incubated in substrate containing DAB (diaminobenzidine, Bio-Rad, Rockville Center, N.Y.) and hydrogen peroxide, and then observed with transmission electron microscopy (Hitachi-500).

4) Agglutination test and growth inhibition test: Agglutination test was performed in a microtiter plate (Haggerty and John, 1982). For the concentration of culture supernatant containing McAb, it was precipitated with saturated $(NH_4)_2SO_4$ in 50% (v/v) and the precipitate was dialysed against distilled water. Thirty microliter of 5×10^4 washed amoebae were added to a 96 well sterile plate containing 100 μl concentrated culture supernatant. The mixture was agitated to promote mixing and incubated at 37°C for 30 mins and evaluated microscopically for agglutination.

To determine the effect of McAbs on proliferation of *N. fowleri*, guinea pig complement (CSL, Australia) was added to final concentration of 5% and the amoebae were incubated for another 30 mins. The plates were placed on ice for 10 mins to dislodge adhered *Naegleria*. Detached amoebae were inoculated into a subculture tube containing CGVS medium and the number of *Naegleria* were counted after 48 hrs (Nash and Aggarwal, 1986).

5) Effects of monoclonal antibodies on cytotoxicity against CHO cells by *N. fowleri*: Chinese hamster ovary (CHO) cell line was selected for assaying the cytopathic effect of free-living amoeba (Lee *et al.*, 1986). Cytotoxicity was determined by the methods of Alderete and Pearlman (1984). Amoeba trophozoites (10^4) and culture supernatant containing McAb were poured on CHO cell monolayer and were incubated for 18 hrs at 37°C CO_2 incubator. The wells were gently washed with EBSS (Earles balanced salt solution). The remaining cells were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet for 10 mins. Excess of the stained material was subsequently washed twice with distilled water and the plates were air dried. The remaining stain solubilized in 0.2% Triton X-100. Individual wells then scanned using an ELISA reader at 570 nm. Cytotoxicity was calculated as follow:

$$\text{Cytotoxicity}(\%)=\left(1-\frac{\text{Absorbance of experimental sample}}{\text{Absorbance of control sample}}\right)\times100$$

6) Enzyme-linked immunoelectrotransfer blot technique (EITB): To find out the molecular weights of antigens binding with McAbs, *N. fowleri* lysate preparations were solubilized with an equal volume of 2× loading buffer (0.125 M Tris HCl pH 6.8, 4% SDS, 20% glycerol) for 5 mins at 100°C. Antigen samples were electrophoresed through 12% polyacrylamide slab gels in the discontinuous buffer system. Electrophoresis was performed at 15 mA in the stacking gel and 20 mA in the separating gel (Laemmli, 1970). Immunoblotting of the gels was performed by the methods of Towbin *et al.* (1979). Electrophoretically separated proteins were transferred to the nitrocellulose paper at 60 volts for 3 hrs. The nitrocellulose sheets were blocked by incubating them with 2% BSA in PBS for overnight at 4°C. The sheets were subsequently incubated for 2 hrs at 37°C with 1 : 1,000 dilutions of ascitic fluid for 2 hrs at 37°C. Thereafter the paper was incubated for 1 hr with peroxidase conjugated goat antimouse immunoglobulins and then exposed for 5~10 mins to the substrate solution which consisted of DAB and H_2O_2. The nitrocellulose sheets were then thoroughly rinsed with water.

RESULTS

1. Isotypes of McAbs: Seven McAbs were

cloned. The isotypes were IgG_1, IgG_3 and IgA. McAb Nf 27 and McAb Nf 154 were IgG_1. McAb Nf 137 was IgG_3. And McAb Nf 1, McAb Nf 2, McAb Nf 256 and McAb Nf 279 were IgA (Table 1).

2. Specificity of anti-*Naegleria fowleri* McAbs: To examine the immunological specificity of these McAbs, ELISA were applied with antigens of *N. fowleri* and other free-living amoeba, such as *N. gruberi*, *N. jadini*, *A. culbertsoni*, *A. royreba*. Except McAb Nf 256, the other McAbs were reacted with *N. fowleri* specifically although McAb Nf 1 was reacted with *N. jadini* and *A. royreba* weakly. McAb Nf 256 was demonstrated positive reaction non-specifically with all five free-living amoebae (Table 2).

3. IFAT and Immunoperoxidase stain: As a result of IFAT, five McAbs (Nf 2, Nf 27, Nf 154, Nf 137 and Nf 279) stained a localized area on the cell membrane whereas McAb Nf 1 stained diffuse area of the cell membrane (Fig. 1). McAb Nf 256 failed to react with *N. fowleri*. From these results six antibodies (except McAbs Nf 256) were specific for antigenic determinants located on the surface membrane.

As a part of experiments, immunoperoxidase staining was also done. The amoebae were exposed to the culture supernatant without McAb of the control group. Peroxidase activity was observed on mitochondria only. When *N. fowleri* trophozoites were exposed to the culture supernatant containing McAb Nf 154, peroxidase

Table 1. Isotype of monoclonal antibodies

Monoclonal antibodies	Isotype
Nf 27, Nf 154	IgG_1
Nf 137	IgG_3
Nf 1, Nf 2, Nf 256, Nf 279	IgA

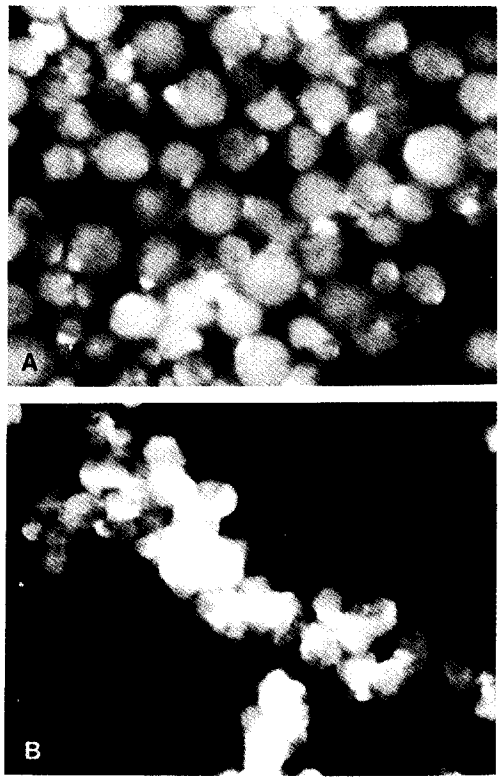

Fig. 1. (A) Indirect immunofluorescence findings of *N. fowleri* trophozoites incubated with McAb Nf 2 showing fluorescence in localized area of cell membrane. (B) McAb Nf 1 showing diffuse fluorescence on the cell membrane.

Table 2. Cross-reactivity of anti-*N. fowleri* monoclonal antibodies with free-living amoeba, measured by ELISA

Free-living amoeba	Monoclonal antibodies						
	Nf 1	Nf 2	Nf 279	Nf 256	Nf 27	Nf 154	Nf 137
N. fowleri	>2.0	1.28	>2.0	0.37	0.81	1.02	1.25
N. gruberi	0.076	<	0.006	0.38	<	0.01	0.001
N. jadini	0.12	0.02	0.03	1.05	0.03	0.04	0.03
A. culbertsoni	<	<	<	0.35	<	<	<
A. royreba	0.11	0.04	0.05	0.5	0.04	0.06	0.04

<; absorbance is lower than 0.001.

activity was observed over the entire amoeba surface and mitochondria (Fig. 2). Binding of McAb Nf 154 was evident from peroxidase activity on the cell surface as compared to the control.

4. Agglutination and growth inhibition test: We tested the antibodies for their ability to agglutinate live *N. fowleri* trophozoites. Five McAbs (Nf 1, Nf 2, Nf 279, Nf 154, Nf 137) agglutinated live *N. fowleri* trophozoites *in vitro*

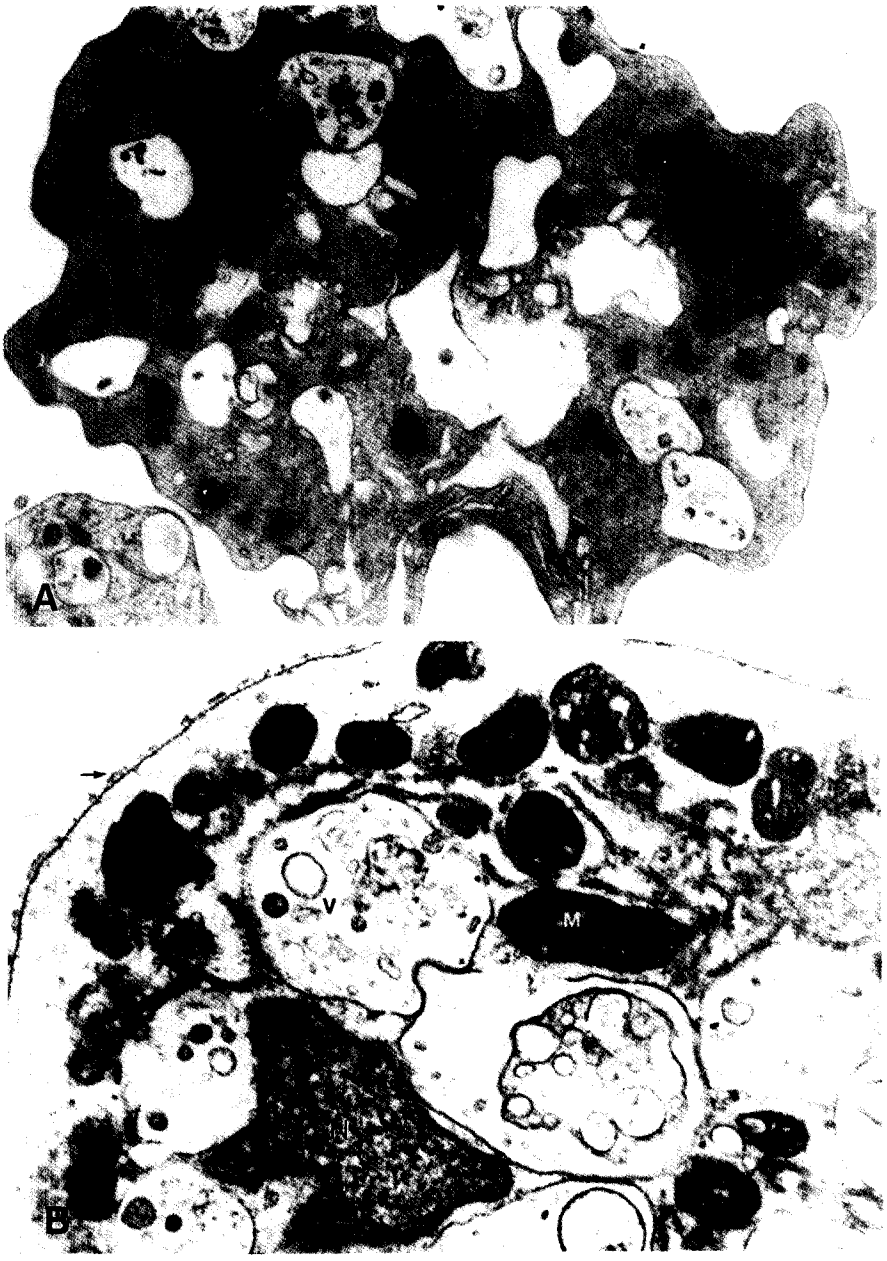

Fig. 2. (A) EM of *N. fowleri* trophozoites incubated with culture supernatant without McAb. Peroxidase activity was demonstrated on mitochondria only. (B) EM of *N. fowleri* trophozoites incubated with McAb Nf 154. Peroxidase activity was observed over the amoeba surface membrane and mitochondria. N; nucleus, M; mitochondria, V; vacuole

(Table 3). When agglutinated *N. fowleri* trophozoites were added to subculture medium, the amoeba proliferation was inhibited(ranges; 0.43 ~0.88) on the basis of control group to be 1. McAb Nf 2 significantly reduced growth of the amoebae(0.43±0.014) (Table 4).

5. **Cytotoxicity of *N. fowleri* against CHO cells:** When *N. fowleri* trophozoites were put on CHO cell monolayer, control cytotoxicity was 64.2%, whereas McAb Nf 2 and Nf 154 added to CHO cell monolayer, cytotoxicities were decreased 11.4% and 25.7% respectively (Table 5).

Table 3. Agglutination of *N. fowleri* trophozoites by anti-*N. fowleri* monoclonal antibodies

Monoclonal antibodies	Agglutination
Nf 1	yes
Nf 2	yes
Nf 279	yes
Nf 27	no
Nf 154	yes
Nf 137	yes

Table 4. Effect of monoclonal antibodies on *in vitro* proliferation of *N. fowleri*

Monoclonal antibodies	Relative No. of *N. fowleri* trophozoites
Nf 1	0.61±0.079*
Nf 2	0.43±0.014**
Nf 279	0.80±0.155
Nf 27	0.88±0.070
Nf 154	0.74±0.056
Nf 137	0.65±0.028*
control	1.00

* $p<0.1$ ** $p<0.05$

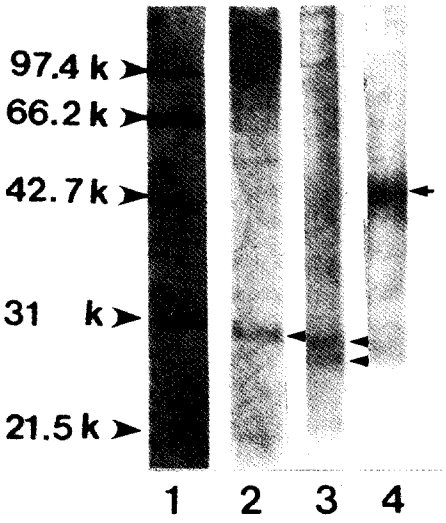

Fig. 3. Enzyme-linked immunoelectrotransfer blotting of *N. fowleri* lysate reacted with various monoclonal antibodies. M.W. protein standards(1), Nf 137(2), Nf 279 (3) and Nf 154(4)

Table 5. Effect of monoclonal antibodies on cytotoxicity against Chinese hamster ovary cells by *N. fowleri*

Monoclonal antibodies	Cytotoxicity(%)
Nf 1	63.1±4.14
Nf 2	11.4±4.75*
Nf 279	66.0±2.35
Nf 256	67.3±2.86
Nf 27	71.4±1.98
Nf 154	25.7±1.79*
Nf 137	65.5±3.04
control	64.2±3.52

* $p<0.05$

Table 6. Summary of characteristics of 7 anti-*N. fowleri* monoclonal antibodies

Characteristics	Monoclonal antibodies						
	Nf 1	Nf 2	Nf 279	Nf 256	Nf 27	Nf 154	Nf 137
Isotype	IgA	IgA	IgA	IgA	IgG_1	IgG_1	IgG_3
IFAT	+*	+	+	−	+	+	+
Agglutination	+	+	+	ND	−	+	+
Growth inhibition	±	+	−	ND	−	−	±
Cytotoxicity inhibition	−	+	−	−	−	+	−
EITB(dalton)			25k, 28k			43k	29k

* diffused

6. **EITB:** For the determination of the molecular weight(MW) of antigens binding with McAbs, immunoblotting was done. The recognized *N. fowlei* antigens were as follows; McAb Nf 279 of 28 kDa and 25 kDa, McAb Nf 137 of 29 kDa, McAb Nf 154 in a single band of approximately 43 kDa(Fig. 3). The other McAbs (Nf 1, Nf 2, Nf 256, Nf 27) did not appear to react with any antigens of *N. fowleri* by immunoblotting.

DISCUSSION

N. fowleri is a pathogenic free-living amoeba which causes acute primary amoebic meningoencephalitis in man or experimental animals. The diagnosis of this protozoan infection has been accomplished only upon postmortem examination by detecting the causative protozoa. However, the species identification of this protozoa is still not easy because the taxonomical criteria to differentiate the morphological characteristics among species have not yet been fully established. This study was attempted to produce the monoclonal antibody specific for *N. fowleri* and to provide information about its characteristics and capability for diagnosis or taxonomy.

The 7 clones secreting McAbs against *N. fowleri*, whose isotypes were IgG_1, IgG_3 and IgA, were produced in the present study. Specificities of the McAbs were tested by using antigens of other *Naegleria* spp. by ELISA. Among the 7 McAb, McAb Nf 2, McAb Nf 279, McAb Nf 27, McAb Nf 154 and McAb Nf 137 reacted only to *N. fowleri*, and did not react to antigens of other free-living amoebae. Those 5 McAbs were regarded as specific to *N. fowleri*. We expect that the 5 anti-*N. fowleri* specific McAbs can be applied to taxonomy of free-living amoebae or to diagnosis of amoebic meningoencephalitis patients by tagging enzymes, fluorescent materials or radioisotope. This should be a subject of further study.

Visvesvara and Healy (1975) and Willaert (1976) analysed the comparative antigenicity of free-living amoebae by immunoelectrophoresis. They reported that the antigenicity of *N. jadini* was 5.2, *N. gruberi* 4.3, *Naegleria* species, 3.9, *Acanthamoeba* spp. less than 1 on the basis of *N. fowleri* to be 10. In spite of common antigenicity between *Naegleria* spp., 5 anti-*N. fowleri* specific McAbs were produced in the pressent study.

To find out the MW of antigens bound by McAbs, whole cell lysate of *N. fowleri* was separated by SDS-PAGE, electroblotted on nitrocellulose, and probed by McAbs. McAb Nf 279, McAb Nf 154 and McAb Nf 137 bound with *N. fowleri* antigens, while the others did not react to any antigens of *N. fowleri* by immunoblotting. The MWs of antigens bound with McAb Nf 279 were 28 kDa and 25 kDa. MWs of McAb Nf 154 and McAb Nf 137 were 43 kDa and 29 kDa, respectively. Bickle *et al.* (1986) reported that MWs of *Schistosoma mansoni* antigens with which most of McAbs reacted had been established either by immunoprecipitation method or by immunoblotting. Since the MWs of antigens with which McAbs(McAb Nf 1, McAb Nf 2, McAb Nf 256, McAb Nf 27) reacted were not determined by immunoblotting, immunoprecipitation method can be the next trial.

IFAT and immunoperoxidase staining with McAbs reacted to the antigens on the surface membrane of *N. fowleri* trophozoites. Six monoclonal antibodies(except Nf 256) reacted to the surface membrane of live *N. fowleri*, and peroxidase activity was detected on the surface membrane of trophozoites by immunoperoxidase stain with McAb Nf 154, which also confirmed the result of IFAT. It was supposed that those six surface membrane reactive McAbs have harmful effect on the motility of *N. fowleri*. Some effective functions of surface membrane reactive monoclonal antibodies to various parasites were studied by a few investigators. Epstein *et al.* (1981) reported that *P. knowlesi* merozoite agglutinating McAbs blocked erythrocyte invasion of the parasite most effectively, and Burgess (1986) reported that a McAb against *Tritrichomonas foetus* faciliated complement mediated lysis

of *T. foetus*. In the present study, to evaluate the effector function of the surface membrane reactive McAbs, their ability to cause agglutination, growth inhibition test and cytotoxicity of *N. fowleri* to CHO cells were examined. Five McAbs(Nf 1, Nf 2, Nf 279, Nf 154, Nf 137) agglutinated live organisms and inhibited proliferation of the amoeba. The McAb Nf 2 significantly reduced the growth of amoeba. McAb Nf 2 and McAb Nf 154 also decreased the cytotoxicity on CHO cells by *N. fowleri* trophozoites. Although the isotype of McAb Nf 2 is IgA, McAb Nf 2 has more stronger effect on weakening virulence of the amoeba than the other McAbs whose isotypes are IgG do. Weiss (1985) described that antibody-dependent cell mediated cytotoxicity reactions might be restricted to certain isotypes in analyzing McAbs for antiparasite activity; *i.e.*, the clearance of *D. viteae* microfilariae in mice was mediated by IgM but not by IgG antibodies(Thompson *et al.*, 1979). Therefore, it is possible that McAb Nf 2 may provide any host protective effect *in vivo*.

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=국문초록=

*Naegleria fowleri*에 대한 단세포군 항체의 생산과 그 특성에 관한 연구

한양대학교 의과대학 기생충학교실, 연세대학교 의과대학 기생충학교실*

류 재 숙 · 임 경 일*

자유생활 아메바인 *Naegleria fowleri*는 비강을 통해 인체에 들어와 출혈성 수막뇌염을 일으켜 감염된 사람의 대부분을 일주일 이내에 사망하게 하는 원충이다. 이 실험에서는 *N. fowleri*에 특이한 단세포군 항체를 만들어 이의 특성 및 그 이용 가능성을 알아보고자 하였다.

7 종류의 단세포군 항체(Nf 1, Nf 2, Nf 256, Nf 279, Nf 27, Nf 154, Nf 137)를 제조하였는데 각각의 isotype은 IgG_1이 두 종류(Nf 27, Nf 154), IgG_3가 1 종류(Nf 137), IgA가 4 종류(Nf 1, Nf 2, Nf 256, Nf 279)이었다. 이들 항체를 *N. fowleri*와 다른 종류의 아메바를 항원으로 하여 효소표식 면역검사법을 시행하여 Nf 1 및 Nf 256 항체를 제외한 5 종류의 항체가 *N. fowleri*에 특이한 항체임을 확인하였다. 또한 간접 형광항체법을 통하여 Nf 256 항체를 제외한 6 종류의 단세포군 항체가 세포막의 일부에 결합하였음을 관찰하였다. Nf 154 항체를 이용한 immunoperoxidase 염색에서도 세포막의 반응을 관찰하였다.

단세포군 항체가 *N. fowleri*를 응집시키는지 알아보고자 항체를 *N. fowleri* 영양형과 반응시켰더니 Nf 27 항체를 제외한 5 종류의 단세포군 항체에 의해 응집이 일어남을 알 수 있었으며 응집된 영양형을 보체로 처리한 후 CGVS 배지에서 배양하였더니 영양형의 증식이 억제되었다. 또한 2 종류의 단세포군 항체(Nf 2, Nf 154)는 조직 세포(Chinese hamster ovary cell: CHO)에 대한 *N. fowleri*의 세포독성을 저하시켰다. 단세포군 항체와 반응하는 항원의 분자량을 알아보고자 EITB(Enzyme-linked immunoelectrotransfer blot)를 시행하였는데 Nf 279 항체는 25 kDa 및 28 kDa, Nf 154 항체는 43 kDa, Nf 137 항체는 29 kDa에 해당되는 분획에서 항원항체반응을 나타내었다.

이상의 성적을 종합하면 *N. fowleri*에 대한 7 종류의 IgG., IgG_3 및 IgA 단세포군 항체를 생산하였다. 그중 Nf 256 항체를 제외한 6 종류는 *N. fowleri*의 세포막 성분중 28 kDa - 43 kDa의 항원과 반응하는 특이한 항체임을 관찰할 수 있었다. 또한 이 단세포군 항체들은 영양형을 응집시키며, 시험관 내에서의 증식을 억제시키고 CHO 세포에 대한 *N. fowleri*의 세포 독성을 저하시키는 성질을 가지고 있음을 알 수 있었다.

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