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Use of Monoclonal Antibodies To Distinguish Pathogenic *Naegleria fowleri* (Cysts, Trophozoites, or Flagellate Forms) from Other *Naegleria* Species

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Monoclonal antibodies (MAbs) reactive to the pathogenic amoeba *Naegleria fowleri* were analyzed by enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay, Western blotting (immunoblotting), and radioimmunoprecipitation assay (RIPA). Two MAbs (3A4 and 5D12) showed reactivity by ELISA with all *N. fowleri* strains tested and no reactivity with the five other *Naegleria* species, *N. lovaniensis*, *N. gruberi*, *N. australiensis*, *N. jadini*, and *N. andersoni*. These MAbs reacted with the three morphological forms of *N. fowleri* (trophozoites, cysts, and flagellates). The reactivity on Western blots was suppressed by treatment with metaperiodate, suggesting a carbohydrate epitope. Differences in reactivity patterns between trophozoites and cysts observed with radioimmunoprecipitation assay might reflect differences in biological properties. The formalin stability of the epitope may be useful in detecting *N. fowleri* in fixed biopsies and in investigating the pathological process.

Among members of the genus *Naegleria*, only *N. fowleri* is pathogenic for humans and produces an acute and fatal meningoencephalitis (3, 6, 7, 8). *N. fowleri* infects mostly young and healthy people who have been swimming in thermally polluted waters. Infection of the nasal mucosa and the central nervous system occurs in a few days and has a dramatic clinical course (5, 6, 7, 9, 11). Identification techniques have to be specific, sensitive, and rapid to differentiate *N. fowleri* encephalitis from *Acanthamoeba*, viral, or bacterial encephalitis.

For environmental monitoring in a preventive objective, we need to distinguish pathogenic *N. fowleri* from nonpathogenic *Naegleria* spp. in water samples and to be sure to identify the three forms of the amoeba (cysts, trophozoites, and flagellates).

Immunological approaches with monoclonal antibodies (MAbs) appear to be a powerful tool. Preliminary results for trophozoite forms have been reported (1, 12). The goal of this study was to produce and characterize specific MAbs to *N. fowleri* strains (including the different morphologic forms, i.e., trophozoites, cysts, and flagellates).

MATERIALS AND METHODS

Amoebae. Nine strains of *N. fowleri*, five strains of *N. lovaniensis*, two strains of *N. gruberi*, one strain of *N. australiensis*, one strain of *N. jadini*, and one strain of *N. andersoni* (Table 1) were grown axenically in Chang's SCGYE medium, containing casein, glucose, yeast extract, and fetal calf serum (10%). Cultures were incubated at 37°C, except for those of *N. gruberi* and *N. jadini*, which were grown at room temperature.

Cell recovery. Exponentially growing trophozoites were harvested following centrifugation at 1,000 × *g* for 15 min. Pellets were suspended in phosphate-buffered saline (PBS), pH 7.2. Cells were counted in a Thoma's counting chamber. A cell suspension containing 10^6 organisms per ml was prepared, and then trophozoites were lysed by thermal shocks with liquid nitrogen.

Immunization of mice. Two female BALB/c mice were immunized intraperitoneally with a mixture of 10^6 washed and killed *N. fowleri* E4A2 trophozoites in Freund's complete adjuvant in a volume of 0.3 ml. After 15 and 30 days, the mice were given an intraperitoneal injection of 0.3 ml of killed amoebae (without Freund's adjuvant). Sera were tested by enzyme-linked immunosorbent assay (ELISA), and 3 days before cell fusion, mice were given a fourth injection (intravenously in the tail).

Cell fusion. Spleens from the two mice were removed. Spleen cells were fused with hypoxanthine guanine phosphoribosyl transferase-negative myeloma (SP-2/0-Ag14) cells (2). Cells were mixed in a proportion of 5:1 (spleen cells to myeloma cells) in the presence of a 50% (wt/vol) solution of polyethylene glycol 4000 (Merck). After washing and centrifugation, the cells were suspended in RPMI culture medium containing 50 μM hypoxanthine, 10 μM aminopterine, and 0.4 μM thymidine (HAT) (GIBCO, Life Technologies Ltd., Paisley, Scotland) supplemented with glutamine (1% [vol/vol]), sodium pyruvate (1% [vol/vol]), and fetal calf serum (20% [vol/vol]), and then dispensed into 96-well microtiter culture plates (Falcon). Cells were maintained at 37°C in a 5% CO_2 atmosphere. After 10 to 15 days of hybrid cell growth, the culture supernatant fluids were screened by ELISA. Hybrid cultures demonstrating an antibody activity were successively grown in 24-well plates and culture flasks and then cloned twice by limiting dilution in 96-well plates.

ELISA. ELISA was performed by seeding 10^4 *N. fowleri* cells (suspended in 100 μl of carbonate buffer) into each well of a 96-well microtiter ELISA plate (Nunc; InterMed, Roskilde, Denmark). The cells were allowed to adsorb overnight at 4°C. Plates were washed three times with PBS containing 1.0% Tween 20 (PBS-T) and then dried. One

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TABLE 1. Strains used in this study

Species and strain	Origin	Yr of isolation
N. fowleri		
E4A2	France	1979
Kul	Belgium (H)[a]	1973
0359	Belgium (H)	1970
Mo4-44	France	1979
Moj31c	France	1987
Moj32b	France	1987
Moj200a	France	1987
Na420c	France	1988
Na1165b	France	1988
N. lovaniensis		
F4	Belgium	1980
F9	Belgium	1980
Ar9Ml	United States	1976
78.76.S9	Belgium	1976
76.15.250	Belgium	1976
N. gruberi		
1518/1e	United States	1964
1518/1f	United States	1965
N. australiensis LSR49	France	1979
N. jadini 0.400	Belgium	1972
N. andersoni jamiesoni T56E	Singapore	1981

[a] H, strain of human origin.

hundred microliters of the supernatant from hybrid cultures was added to each well. Plates were incubated for 30 min at room temperature and washed three times with PBS-T. One hundred microliters of a 1:10,000 dilution of peroxidase-conjugated affinity-purified rabbit anti-mouse immunoglobulin G (IgG) (Jackson; ImmunoResearch, Paris, France) was added to each well. The plates were incubated for another 30 min at room temperature, washed three times in PBS-T, and developed for 15 min in a phosphate-citrate buffer (pH 5.0) with 0.3% (wt/vol) *o*-phenylenediamine and 0.15% (vol/vol) perhydrol (final concentrations). Reactions were stopped with 0.05 M sulfuric acid. Optical densities at 492 nm were measured on an LP200 apparatus (Diagnostics Pasteur, Marnes la Coquette, France).

Indirect immunofluorescence microscopy (IIF). Approximately 10^4 trophozoite or flagellate forms were placed onto glass slides with a pipette and incubated at room temperature for 30 min. The temporary flagellate forms were obtained by transferring trophozoites from growth medium to distilled water after 3 h at 37°C. The organisms were fixed to the glass in 5% formalin. After 15 min, supernatant fluids were removed and the organisms were then covered with supernatant fluids from hybrid cultures. The slides were incubated in a moist chamber for 1 h at 37°C, washed in PBS, and then covered with 40 μl of a 1:100 dilution of fluorescein-conjugated rabbit anti-mouse IgG (Diagnostics Pasteur). After incubation for 1 h in a moist chamber at 37°C, the slides were washed with PBS and coverslips were mounted with a glycerol mounting fluid (Fluoprep; Biomerieux, Marcy l'Etoile, France). The slides were examined for fluorescence with an Axioscop fluorescence microscope (Zeiss, Oberkochem, Germany). Appropriate controls were performed to avoid nonspecific fluorescence or autofluorescence of organisms.

Radioimmunoprecipitation assay (RIPA). Tests were performed with cultures from three *N. fowleri* strains (Moj32b, Moj200a, and Moj420c) and two *N. lovaniensis* strains (76.15.250 and Ar9Ml).

(i) RIPA with trophozoites. Amoebae were grown on Chang's medium supplemented with [^{35}S]methionine (200 μCi in 3 ml of medium). After 3 days of incubation at 37°C, the organisms were removed, suspended in PBS, and then centrifuged at 1,000 × *g* for 15 min. The pellet was suspended in 50 μl of 1× RIPA buffer (0.15 M NaCl, 0.05 M Tris-HCl [pH 7.5], 1 mM EDTA, 1% [wt/vol] Triton X-100, 1% [wt/vol] sodium desoxycholate, 1% [wt/vol] phenylmethylsulfonylfluoride, 0.1% [wt/vol] sodium dodecyl sulfate [SDS]) and lysed with liquid nitrogen. After centrifugation at 70,000 × *g* for 15 min, 50 μl of amoebic supernatant was mixed with an equal volume of MAb fluid for 2 h at 4°C. Next, 10 μl of M-450 dynabeads coated with sheep anti-mouse IgG (Biosys, Compiegne, France) was added. After 1 h of incubation at 4°C, the dynabeads were washed in 1× RIPA buffer. Immune complexes were separated from beads after centrifugation in denaturing sample buffer (0.0625 M Tris [pH 6.8], 1% [wt/vol] SDS, 10% [vol/vol] glycerol, 1% [vol/vol] β-mercaptoethanol, 0.01% [wt/vol] bromophenol blue) before being loaded on gels.

(ii) RIPA with encysted cells. Cultures were developed on 1% nonnutrient agar plates (55 mm diameter) in the presence of *Escherichia coli*. Cysts from an old culture were placed in the center of the plates inoculated with bacteria. After 24 h, the growing trophozoites were surrounded with 125 μl of distilled water containing [^{35}S]methionine (500 μCi). As cultures were developing, the organisms moved excentrically through the radiolabeled zone. The plates were maintained at 37°C until encystment was complete. Cysts were removed in PBS, and treatment with 1× RIPA buffer was done as described above.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on vertical slab gels containing 12.5% acrylamide as described previously (4) (Slab Gel Electrophoresis SE 250 apparatus; Hoefer Scientific Instruments, San Francisco, Calif.). Gels were loaded with 15 μl (final concentration of 1 μg of protein per μl) of each sample and electrophoresed for 1 h at 150 V.

Autoradiography. Gels were dried for 45 min in a thermal Speed Vac and covered with a Kodak XAR X-ray autoradiographic film.

Western blotting (immunoblotting). Gels obtained as described above were transferred onto nitrocellulose membranes for 30 min at 300 mA. Membranes were cut and incubated overnight at room temperature in 1% bovine serum albumin solution. Strips were washed three times in PBS-T, incubated overnight at room temperature with nondiluted hybrid supernatant, and then washed three times in PBS-T. After 1 h of incubation at room temperature with a 1:1,000 dilution of anti-mouse IgG peroxidase conjugate (Jackson; ImmunoResearch), strips were washed with PBS-T and bands were visualized in a solution containing 5 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.) and 10 μl of 30% H_2O_2 in 100 ml of PBS. Reactions were stopped by the addition of 0.05 M HCl, and membranes were washed in distilled water and then dried at room temperature.

Periodate oxidation. Nitrocellulose membranes were incubated for 1 h at room temperature with a sodium metaperiodate solution from 0.1 to 100 mM before being incubated with conjugate as described previously (12).

TABLE 2. ELISA and IIF results

Species and strain	Mean OD (SD) with MAb[a]	
	3A4	5D12
N. fowleri		
E4A2	100 (8.2)	100 (5.2)
0359	80.1 (2.7)	66.7 (10.9)
Mo4-44	97.5 (8.9)	88.2 (4.4)
Moj32b	90.7 (2.2)	94.6 (11.8)
KUL	97.7 (10.0)	88.7 (5.7)
Moj31c	95.0 (10.3)	102.3 (3.7)
N. lovaniensis		
F4	3.5 (0.1)	4.2 (<0.1)
F9	3.8 (0.4)	4.3 (0.4)
78.76.S9	3.7 (0.1)	4.2 (<0.1)
76.15.250	3.7 (<0.1)	4.1 (0.2)
N. gruberi 1518/1e	3.6 (0.3)	4.0 (0.4)
N. australiensis LSR49	3.8 (0.1)	4.3 (0.3)
N. andersoni jamiesoni T56E	3.7 (0.3)	4.2 (0.3)
N. jadini 0.400	3.8 (0.2)	4.1 (0.4)

[a] Serotypes for MAbs 3A4 and 5D12 were IgG2a κ and IgG1 κ, respectively; IIF results were strongly positive for both MAbs. Results are averages for two assays, with an OD (optical density) at 492 nm of 100 for the immunizing strain E4A2.

RESULTS

Examination of 96-well plates revealed growth of hybrid cells in 790 wells (97.7%). Among them, 189 wells exhibited antibody activity and these cultures were developed in 24-well plates. Next, 35 hybrid cultures showing a strong reactivity by ELISA were developed in 50-ml flasks. Antibodies generated by four clones (2B6, 3A4, 5D12, and 9C10) reacted by ELISA with each strain of *N. fowleri* tested and did not react with any of the *N. lovaniensis* strains tested (data not shown). Two of the four clones (3A4 and 5D12) secreted IgG2a and IgG1, respectively, and were used for further study. These two clones did not react with *N. lovaniensis*, *N. gruberi*, *N. andersoni*, and *N. jadini* strains (Table 2).

A further testing of 3A4 and 5D12 antibodies was performed by IIF on *N. fowleri* E4A2 trophozoites and *N. fowleri* Na 1165b cysts or flagellate forms. With trophozoites, results were strongly positive. Undiluted supernatants of 3A4 and 5D12 MAbs led to an immunofluorescent staining of the whole cell (Fig. 1A and B). When the MAbs were used diluted, the reactivity appeared to be located at the cell surface. IIF was negative with other tested *Naegleria* spp. (i.e., *N. gruberi* and *N. lovaniensis*) (Fig. 1E).

With the flagellate forms, results were also positive with the two MAbs (Fig. 1C and D). A cell surface fluorescence was observed. IIF with cysts showed only peripheral labeling.

Western blots performed with trophozoites and cysts were similar, and these two forms exhibited identical profiles with the two MAbs (Fig. 2). The observed pattern showed a heterogeneous reactivity, with multiple components at molecular masses ranging between 20 and 90 kDa, similar to the washboard pattern observed with polysaccharides. The reactivity was lost after periodate oxidation (Fig. 3).

The RIPA was positive for the cyst form of three *N. fowleri* strains (Moj420c, Moj200a, and Moj32b) and negative for the cysts of two strains of *N. lovaniensis* (76.15.250 and Ar9Ml). Protein profile patterns showed that MAbs 3A4 and 5D12 reacted with *N. fowleri* and not with *N. lovaniensis*. The three protein profile patterns obtained with *N. fowleri* strains demonstrated major protein bands in common with molecular masses of 97,000, 95,000, 50,000, 36,000, 34,000, and 20,000 Da. Some minor protein bands in the range of 30,000 and 46,000 Da seemed to exist (Fig. 4).

The RIPA with trophozoite forms was negative with the two MAbs, indicating that the components recognized were not able to incorporate methionine.

DISCUSSION

Previous works described MAbs against *N. fowleri*, but these studies were performed only on trophozoite forms. Visvesvara et al. (12) obtained MAbs which reacted with cellular components at molecular masses ranging between 34,000 and 97,000 Da, but they did not explain the heterogeneity or chemical features of the recognized epitopes. The study by Flores et al. (1) was performed with *N. fowleri* and *Acanthamoeba* spp., which are not closely linked phylogenetic species. Flores et al. did not characterize their MAbs, but they found that such antibodies may be used for fluorescence flow cytometry, demonstrating a potential use in direct identification of *N. fowleri*.

The purpose of this study was to set up a panel of MAbs to distinguish the pathogenic *N. fowleri* from other *Naegleria* spp. Among the four clones obtained, two (3A4 and 5D12) were selected on the basis of their high affinity as determined by ELISA and were studied by three other techniques (IIF, Western blotting, and RIPA). We tried to determine the specificities of the corresponding epitopes, both in trophozoites and cystic forms.

We have demonstrated that the two MAbs 3A4 and 5D12 did react with *N. fowleri* trophozoites, cysts, or flagellate forms without reacting with *N. lovaniensis* or other *Naegleria* species. The biochemical characterization of the component recognized by the two species-specific MAbs seemed to us very useful to obtain a better understanding of the cell surface structure. The washboard pattern observed in the Western blot assay, as well as the loss of reactivity after periodate oxidation, led us to conclude that the MAb recognized a carbohydrate epitope, shared by trophozoites and cysts.

We tried to check these results by using a RIPA after the metabolic incorporation of [^{35}S]methionine in the amoebic proteins. The negative RIPA observed with actively dividing trophozoites might indicate that the MAbs did not recognize a protein. On the other hand, when RIPA was performed on encysted organisms, a complex pattern exhibiting multiple bands was seen. So the differences in reactivity patterns determined by RIPA between the two amoebic forms were questionable.

First, the negative RIPA obtained with the trophozoites could be due to the fact that the actively dividing forms were synthesizing and turning over proteins so rapidly in the three days of labeling that the amoebae exhausted the [^{35}S]methionine available, leaving no radiolabel to label the proteins synthesized on day 3.

Second, it was also possible that [^{35}S]methionine was much more diluted in Chang's medium than on agar plates and would not be readily incorporated in the trophozoites growing in liquid medium.

Third, the negative RIPA might indicate that the recognized epitope was processed between the trophozoite and

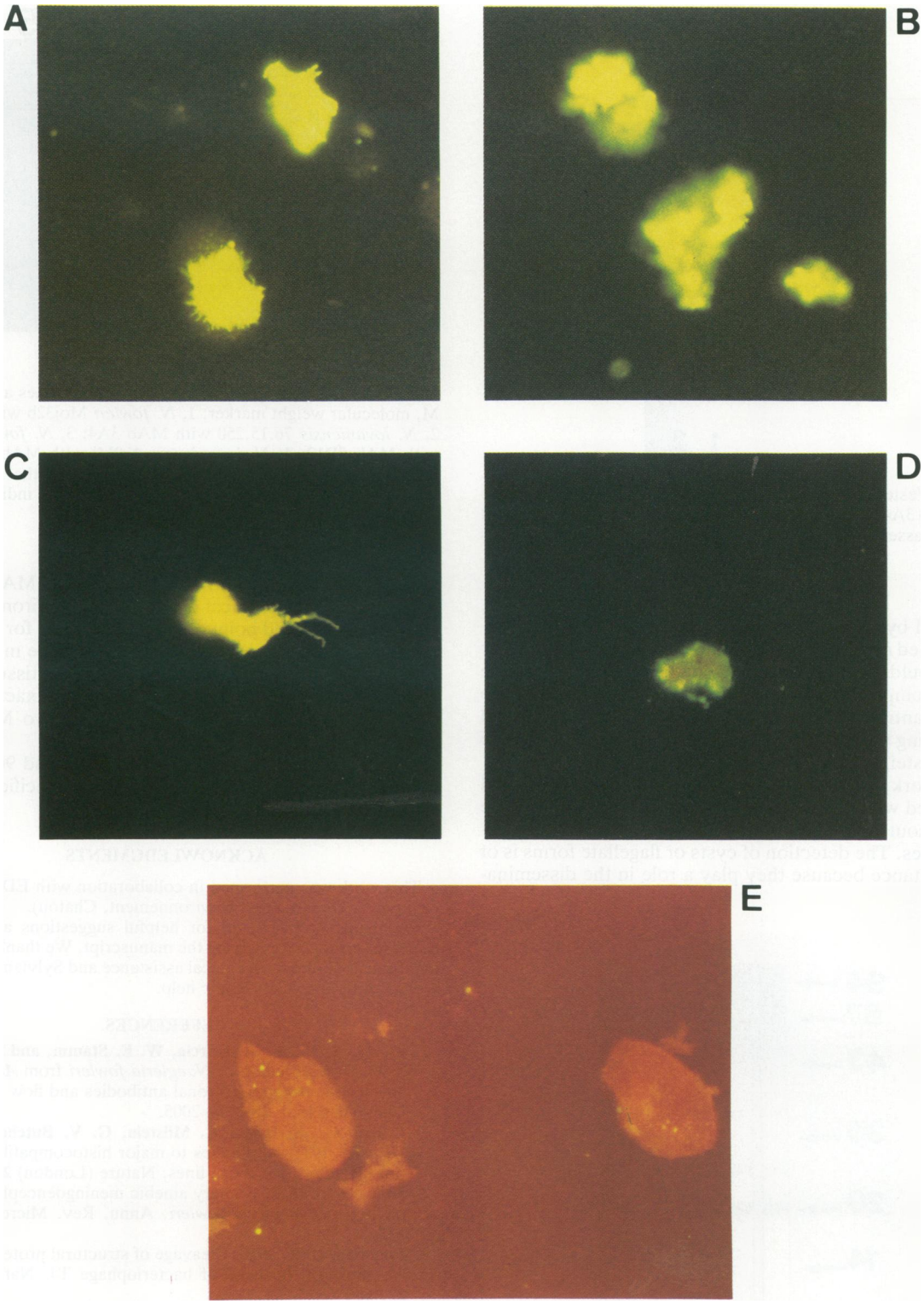

FIG. 1. IIF staining. Trophozoites of *N. fowleri* E4A2 with MAb 3A4 (A) and with MAb 5D12 (B), flagellate forms of *N. fowleri* Na1165b with MAb3A4 (C) and with MAb 5D12 (D), and a trophozoite of *N. gruberi* 1518/1e with MAb 3A4 (E) are shown.

cyst forms. Since the carbohydrate epitope was present both in trophozoites and in cysts in the form of polysaccharide, as demonstrated by Western blot assay, it could be linked on protein components, which were able to incorporate [^{35}S] methionine during the excystation process. These results would suggest a transformation of the antigenic determinant, giving evidence for another phenotypic difference between trophozoites and cysts. *N. fowleri* reportedly is known to be

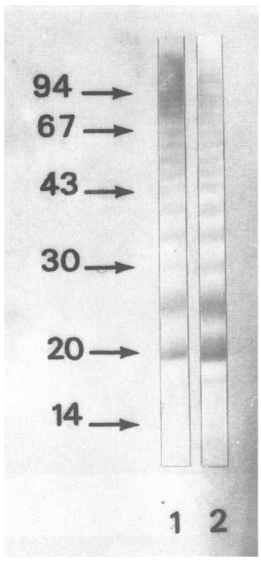

FIG. 2. Western blots with trophozoites of *N. fowleri* KUL. Lane 1, MAb 3A4; lane 2, MAb 5D12. Numbers to the left represent molecular masses in kilodaltons, and arrows indicate the various components.

agglutinated by wheat germ lectin, indicating that this species contained *N*-acetylglucosamine residues (6, 10). Further studies should be performed to characterize the precise chemical composition of the recognized epitope, and to check the antigenic processing between trophozoites and cysts by using various in vitro labels (i.e., [^{14}C]glucosamine and [^{35}S]cysteine).

In this work, we have demonstrated that MAbs 3A4 and 5D12 reacted with *N. fowleri* trophozoite, cyst, or flagellate forms, without reacting with *N. lovaniensis* or other *Naegleria* species. The detection of cysts or flagellate forms is of great importance because they play a role in the dissemination of *Naegleria* spp. in the environment. MAbs could be powerful tools for clinical diagnosis and environmental control, and they could point out specific targets for neutralizing infection. The Formol stability of the epitope may be useful in investigating pathological processes in tissue biopsies. Experiments are in progress to determine exactly the location of the epitope recognized by these two MAbs at the amoebic cell surface.

Further studies with other MAbs (5B6 and 9C10) will be continued to obtain a larger panel of specific antibodies against *N. fowleri* strains.

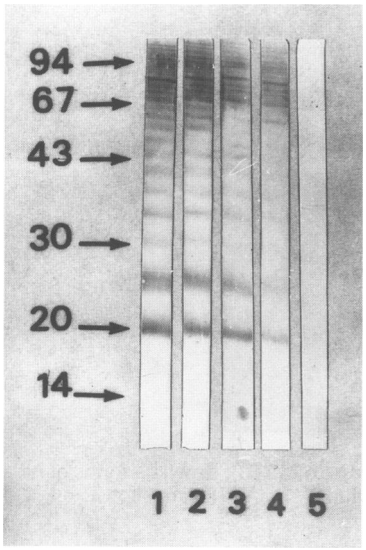

FIG. 3. Oxidation assay with 0, 0.1, 1, 10, and 100 mM metaperiodate (lanes 1, 2, 3, 4, and 5, respectively). Numbers to the left represent molecular masses in kilodaltons, and arrows indicate the various components.

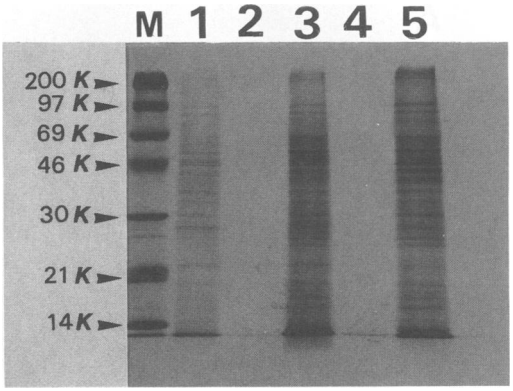

FIG. 4. Autoradiogram with cystic forms. Lanes are as follows: M, molecular weight marker; 1, *N. fowleri* Moj32b with MAb 3A4; 2, *N. lovaniensis* 76.15.250 with MAb 3A4; 3, *N. fowleri* Moj200a with MAb 5D12; 4, *N. lovaniensis* Ar9Ml with MAb 5D12; 5, *N. fowleri* Moj420c with MAb 5D12. Numbers to the left represent molecular masses in kilodaltons, and arrowheads indicate the various components.

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