CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, July 1997, p. 409–414 Vol. 4, No. 4
1071-412X/97/$04.00+0

Preparation of a Monoclonal Antibody Specific for *Entamoeba dispar* and Its Ability to Distinguish *E. dispar* from *E. histolytica*

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Received 24 December 1996/Returned for modification 24 February 1997/Accepted 27 March 1997

A monoclonal antibody (MAb), MAb ED17 (immunoglobulin G2a [IgG2a]), prepared against trophozoites of *Entamoeba dispar* SAW1734RclAR cultured monoxenically with *Crithidia fasciculata*, reacted with 25 of 26 isolates of *E. dispar* by an indirect fluorescent-antibody test. In contrast, the MAb failed to react with any of 20 isolates of *E. histolytica* or other enteric protozoan parasites. Western blot (immunoblot) analysis showed that the molecular mass of the *E. dispar* antigen recognized by the MAb was 160 kDa under reduced conditions. Immunoelectron microscopy revealed that the antigen was mainly located on digested *C. fasciculata*, but not on undigested organisms. Double staining with a mixture of MAb ED17 and MAb 4G6 (an IgG1 MAb which reacts exclusively with *E. histolytica*), followed by incubation with a mixture of fluorescein isothiocyanate-labeled anti-mouse IgG2a and tetramethylrhodamine isothiocyanate-labeled anti-mouse IgG1 antibodies, simultaneously identified mixed populations of *E. dispar* and *E. histolytica*. This method may prove to be useful for the accurate identification of *E. dispar* and *E. histolytica*, even in mixed infections.

Amebiasis, caused by infection with *Entamoeba histolytica*, is one of the most important parasitic diseases not only in developing countries but in developed countries as well. It has been estimated that *E. histolytica* causes 50 million cases of amebic colitis and liver abscess, resulting in at least 40,000 deaths annually (33). *E. histolytica* has recently been reclassified into two species, *E. histolytica* Schaudinn, 1903, and *Entamoeba dispar* Brumpt, 1925, on the basis of biochemical, immunological, and genetic findings (7). The two species are morphologically inseparable, but only *E. histolytica* is the causative agent of invasive amebiasis. Therefore, it is important to distinguish between *E. histolytica* and *E. dispar* for clinical and epidemiological reasons.

The use of monoclonal antibodies (MAbs) is one of the integral parts of a specific and sensitive diagnostic strategy. To date, a number of MAbs which are specifically reactive with *E. histolytica* have been produced (9, 19, 23, 25, 26, 30). MAb 4G6, recognizing a 30-kDa nucleic antigen, was shown to be reactive with various isolates of *E. histolytica* by an indirect fluorescent-antibody test (IFA), regardless of culture conditions, geographic origins, or zymodeme patterns (5, 25, 28). It was also reported that MAbs to a 170-kDa amebic lectin were able to detect *E. histolytica* antigen in feces and serum by enzyme-linked immunosorbent assay (1, 10). In contrast, the production of only one MAb specific for *E. dispar* has been reported (17). Identification of *E. dispar*-specific antigens and/or epitopes is important not only for diagnostic purposes but also for understanding the nature of noninvasive commensal amebae.

Here we report on the preparation and characterization of a MAb specific for *E. dispar* and its application for differentiating *E. dispar* from *E. histolytica*.

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MATERIALS AND METHODS

Parasites and culture conditions. Three culture systems were used: axenic (absence of organisms), useful for *E. histolytica*; monoxenic (one organism), useful for *E. dispar*; and xenic (two or more organisms), useful for *E. dispar* and *E. histolytica*. Trophozoites of *E. dispar* SAW1734RclAR, donated by L. S. Diamond, were cultured monoxenically with promastigotes of *Crithidia fasciculata* in BI-S-33 medium (8) or with *Pseudomonas aeruginosa* in modified BI-S-33 medium (12a). In the latter medium, glucose was replaced with maltose, 0.4% acetone was added, and sterilization was performed by filtration. Trophozoites of *E. dispar* SAW1719, provided by P. G. Sargeaunt, were cultured xenically in the medium described by Robinson (21). Trophozoites of *E. histolytica* reference strains (strains HM-I:IMSS, HK-9, Rahman, 200:NIH, HB-301:NIH, H-302:NIH, H-303:NIH, DKB, C-3-2-1, SAW1627, and SAW755CR) and trophozoites of *Entamoeba moshkovskii* Laredo were axenically grown in BI-S-33 medium. These amebae, except for SAW1627, donated by P. G. Sargeaunt, were also provided by L. S. Diamond. Trophozoites of various amebic isolates were collected and xenically cultured in the medium described by Robinson (21). Some isolates were also monoxenically cultured in BI-S-33 medium with promastigotes of *C. fasciculata* or with epimastigotes of *Trypanosoma cruzi*. Identification of species was by zymodeme analysis (22) and PCR analysis (27). Trophozoites of *Entamoeba hartmanni*, *Entamoeba coli*, *Endolimax nana*, *Dientamoeba fragilis*, and *Trichomonas hominis*, isolated in our laboratories, were also xenically cultured in the medium described by Robinson (21). Trophozoites of *Giardia intestinalis* Portland I were grown in modified BI-S-33 medium (12). Trophozoites grown in the medium described by Robinson (21) were partially isolated with 75% Percoll, as described previously (25). All parasite suspensions were washed three times with ice-cold 10 mM phosphate-buffered saline (PBS; pH 7.4) before being used.

Production of MAbs. Six-week-old female BALB/c mice were inoculated intraperitoneally with 4×10^5 sonicated trophozoites of *E. dispar* SAW1734RclAR and cocultured *C. fasciculata* in Freund's complete adjuvant and were again inoculated after 2 weeks. After an additional 2 weeks, the mice received only washed *E. dispar* and *C. fasciculata*, without adjuvant. Three days later, spleen cells of the immunized mice were isolated and fused with X63 Ag8.653 mouse myeloma cells by 50% polyethylene glycol 1500 (13). Hybridomas secreting MAbs against *E. dispar* were screened by IFA and were subsequently cloned by limiting dilution. Ascites rich in MAbs were obtained by the intraperitoneal inoculation of hybridomas into pristane-primed mice. One of the MAbs, MAb ED17, was used in this study. The MAb was typed as immunoglobulin G2a (IgG2a) with kappa light chains by double diffusion in a gel.

IFA procedure. IFA was carried out as described previously (25). Briefly, trophozoites were fixed with 3% formalin in PBS, washed, and coated onto the wells of multispot glass slides. After blocking nonspecific binding sites with 3% skim milk in PBS, the wells were incubated with 50 μl of MAb solution for 30 min. For the purpose of titration, dilution of MAb ED17 (in ascitic fluid) was started at a ratio of 1:20, followed by twofold stepwise dilutions. Fluorescein isothiocyanate-labeled goat anti-mouse IgG (Medical & Biological Laboratories,

Nagoya, Japan) was used as the second antibody. Normal mouse serum served as a negative control. For double staining, the *E. histolytica*-specific MAb (MAb 4G6) was used (25). Equal numbers of trophozoites of *E. dispar* SAW1734RclAR and *E. histolytica* HM-1:IMSS were mixed, fixed, and coated onto the wells of slides. After blocking, the antigen was incubated with 50 μl of a mixture of MAbs ED17 and 4G6 (1:20 dilution each) for 30 min, followed by incubation with 25 μl of a mixture of fluorescein isothiocyanate-labeled anti-mouse IgG2a and tetramethylrhodamine isothiocyanate-labeled anti-mouse IgG1 antibodies (The Binding Site Ltd., Birmingham, England), each diluted 1:20 in PBS-skim milk, for 30 min. After washing, the slide was observed with a Nikon fluorescence microscope by using a blue or green excitation filter. Color film was double-exposed by using both filters.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot analysis. Trophozoites of *E. dispar* were solubilized with an equal volume of the sample buffer (14) containing 2 mM phenylmethylsulfonyl fluoride, 2 mM *N*α-ρ-tosyl-L-lysine chloromethyl ketone, 2 mM ρ-hydroxymercuriphenyl sulfonic acid, and 4 μM leupeptin for 5 min at 95°C. The supernatant obtained following centrifugation was subjected to electrophoresis. As a molecular mass marker, "DAIICHI" · II (Daiichi Pure Chemicals Co., Tokyo, Japan) was used. Western immunoblot analysis was performed essentially as described previously (25) and was based on the procedure of Towbin et al. (31). Horseradish peroxidase-labeled goat anti-mouse IgG (Organon Teknica Co., Durham, N.C.) was used as the second antibody. Color development was performed by using a Konica Immunostaining HRP-1000 (Konica Co., Tokyo, Japan) color developer. To examine for the presence of carbohydrate residues on the epitope, periodate oxidation was carried out by the method of Woodward et al. (34). Briefly, the blotted membrane was cut into strips and the strips were rinsed with 50 mM sodium acetate buffer (pH 4.5). One strip was exposed to 10 mM sodium metaperiodate in 50 mM acetate buffer for 1 h, in the dark, at 23°C. Another strip, as a control, was incubated with buffer only. The strips were then washed with the buffer described above and were exposed to 50 mM sodium borohydride in PBS for 30 min. After three washes with PBS containing 0.05% Tween 20, the strips were subjected to immunoanalysis as described above.

Immunoelectron microscopy. Trophozoites were fixed in a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in 150 mM sodium cacodylate buffer (pH 7.4) at 4°C for 1 h. Trophozoites were washed in cold 100 mM cacodylate buffer and were then dehydrated in a graded ethanol series and embedded in LR White (London Resin Co. Ltd., Basingstoke, England) according to the manufacturer's recommendation. Polymerization was carried out at 48°C for 24 h. Thin sections were placed on Formvar-coated grids, and the grids were preincubated in 0.5% skim milk in PBS for 5 min. The grids were then incubated with MAb ED17, diluted 1:20, in 0.5% skim milk–PBS for 8 h at 4°C. After rinsing, ultrathin sections were incubated with protein A conjugated to 20-nm gold particles (Zymed Laboratories, South San Francisco, Calif.) diluted 1:20 in 0.5% bovine serum albumin for 1 h, washed, and fixed with 2% glutaraldehyde for 10 min. The sections were rinsed, counterstained with uranyl acetate and lead citrate, and observed with a Hitachi HU-12AS electron microscope.

RESULTS

Specificity of MAb ED17. The reactivity of MAb ED17 to various enteric protozoa was examined by IFA. Pooled ascitic fluids showed a positive fluorescence on trophozoites of *E. dispar* SAW1734RclAR up to a 20,480-fold dilution. In contrast, ED17 did not react with the trophozoites of *E. histolytica* HM-1:IMSS, *E. coli*, *E. hartmanni*, *E. moshkovskii* Laredo, *E. invadens*, *E. nana*, *D. fragilis*, *T. hominis*, or *G. intestinalis*, even with 20-fold dilutions. The fluorescence of trophozoites of *E. dispar* treated with ED17 was located in the cytoplasm (Fig. 1). ED17 did not react with cocultured *C. fasciculata*.

Various isolates of *E. dispar* and *E. histolytica*, maintained in our laboratories, were also tested by IFA (Table 1). ED17 reacted with 25 of 26 *E. dispar* isolates, regardless of their geographic origin, the culture conditions, or zymodemes (isoenzyme profile). Only one isolate (NOT-68) failed to react with the MAb, under both xenic and monoxenic culture conditions. ED17 did not react with any of 20 *E. histolytica* isolates.

Characterization of the antigen recognized by MAb ED17. To localize the epitope recognized by MAb ED17, immunoelectron microscopy was performed. Gold particles were observed primarily on *C. fasciculata* undergoing digestion within trophozoites (Fig. 2a and b, arrows), but not on undigested organisms (Fig. 2a, arrowhead). Labeling of the surface of trophozoites by gold particles did not occur. When *E. dispar* monoxenically cultured with *P. aeruginosa* was tested, gold particles were also located mainly on bacteria undergoing digestion (Fig. 2c, arrows).

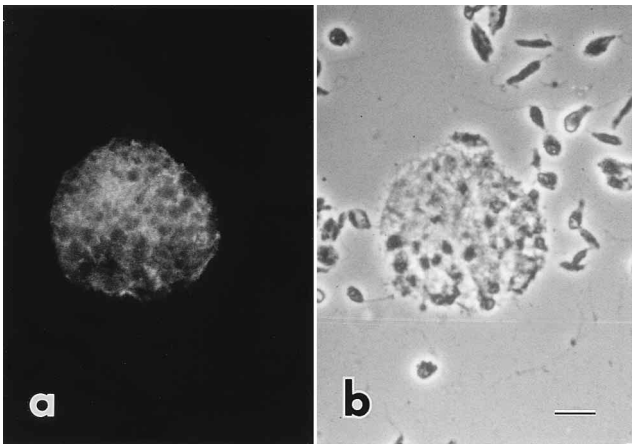

FIG. 1. Immunofluorescence (a) and phase-contrast (b) photomicrographs of *E. dispar* SAW1734RclAR cultured monoxenically with *C. fasciculata*. Formalin-fixed trophozoites were incubated with MAb ED17 (1:20-diluted ascitic fluid) followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG. Bar, 10 μm.

To evaluate the molecular mass of the antigen recognized by MAb ED17, Western immunoblot analysis was carried out (Fig. 3a). A 160-kDa band, isolated in a gradient gel from *E. dispar* trophozoites monoxenically cultured with *P. aeruginosa*, was found to react with ED17. The reactivity of ED17 against the 160-kDa antigen was lost after periodate oxidation, indicating that carbohydrate residues were included in the epitope (Fig. 3b).

Double staining of *E. dispar* and *E. histolytica* by MAbs. Trophozoites of *E. dispar* and *E. histolytica* were mixed and tested by IFA with a mixture of MAbs ED17 and 4G6 (specific for *E. histolytica*). After secondary incubation with a mixture of fluorescein isothiocyanate-labeled anti-mouse IgG2a and tetramethylrhodamine isothiocyanate-labeled anti-mouse IgG1 antibodies, *E. dispar* and *E. histolytica* expressed distinguishing labels (Fig. 4).

DISCUSSION

In the present study, MAb ED17, prepared against *E. dispar* cultured monoxenically with *C. fasciculata*, specifically reacted with *E. dispar* isolates, regardless of their geographic origin, the culture conditions, or zymodemes, but did not react with any other enteric protozoan, including *E. histolytica*. The antigen recognized by ED17 is a 160-kDa glycoprotein. The production of an MAb specific for *E. dispar* has been reported only by Mirelman et al. (17). However, it was recently observed that the MAb also reacted with a xenically cultured *E. histolytica* isolate obtained from a liver abscess (4). Since reactivity was abolished upon axenization of the isolate, they suggested that *E. histolytica* can express the *E. dispar* antigen under certain growth conditions. Although the possibility that the 160-kDa antigen also exists in *E. histolytica* cannot be ruled out at present, the epitope recognized by MAb ED17 seems to be specific for *E. dispar*.

E. dispar is now considered a commensal ameba that is not involved in invasive amebiasis. However, we recently isolated *E. dispar* not only from asymptomatic cyst passers but also from patients with diarrhea and/or bloody stools in northeastern Brazil (28). Since antibodies to *E. histolytica* were not detected, these symptoms cannot be ascribed to amebic infec-

TABLE 1. Reactivity of MAb ED17 to various isolates of *E. dispar* and *E. histolytica*

Species[a]	Isolate	Geographic origin	Culture condition(s)[b]	Zymodeme	IFA[c]
E. dispar	SAW1734RclAR	Israel	Monoxenic	I (III)[d]	+
	SAW1719	Australia	Xenic	I	+
	NOT-23	Peru	Xenic, monoxenic	I	+
	NOT-27	Mexico	Xenic	I	+
	NOT-34	Bolivia	Xenic	I	+
	NOT-35	Peru	Xenic	I	+
	NOT-36	Paraguay	Xenic	I	+
	NOT-38	Mexico	Xenic	I	+
	NOT-67	Indonesia	Xenic	I	+
	SH-3	China	Xenic	I	+
	SH-5	China	Xenic	I	+
	SH-6	China	Xenic	I	+
	MBB-10	Philippines	Xenic	I	+
	MBB-12	Philippines	Xenic	I	+
	AM-13	Brazil	Xenic	I	+
	AM-14	Brazil	Xenic	I	+
	CA-2:NIH	Kenya	Xenic	I	+
	CA-4:NIH	Kenya	Xenic	I	+
	CA-8:NIH	Kenya	Xenic	I	+
	AM-9	Brazil	Xenic	VIII	+
	NOT-33	Cambodia	Xenic	VIII	+
	CA-5:NIH	Kenya	Xenic	IX	+
	CA-6:NIH	Kenya	Xenic	IX	+
	AM-2	Brazil	Xenic	XVII	+
	AM-10	Brazil	Xenic	XVII	+
	NOT-68	Mozambique	Xenic, monoxenic	I (IX)	−
E. histolytica	HM-1:IMSS	Mexico	Axenic	II	−
	HK-9	Korea	Axenic	II	−
	200:NIH	United States	Axenic	II	−
	HB-301:NIH	Burma	Axenic	II	−
	H-302:NIH	Mexico	Axenic	II	−
	H-303:NIH	Vietnam	Axenic	II	−
	DKB	England	Axenic	II	−
	Rahman	England	Axenic	II	−
	C-3-2-1		Axenic	II	−
	NOT-6	Japan	Monoxenic	II	−
	SAW1627	India	Axenic	IIα-	−
	NOT-25	Vietnam	Xenic	VII	−
	NOT-13	Japan	Xenic	XI	−
	SAW755CR	Egypt	Axenic	XIV	−
	SAW1453	India	Xenic	XIV	−
	NOT-3	Japan	Xenic, monoxenic	XIV	−
	NOT-1	Japan	Xenic, monoxenic	XIX	−
	NOT-12	Japan	Monoxenic	XIX	−
	NOT-26	Nepal	Xenic	XIX	−
	NOT-31	India	Xenic	XIX	−

[a] Species determined by PCR and zymodeme analysis.
[b] Monoxenic, cultured monoxenically with promastigotes of *C. fasciculata* or epimastigotes of *T. cruzi* in BI-S-33 medium or with *P. aeruginosa* in modified BI-S-33 medium; Xenic, cultured xenically in the medium described by Robinson (21); Axenic, cultured axenically in BI-S-33 medium.
[c] MAb (ascitic fluid) was used at a 1:20 dilution.
[d] Original zymodemes are indicated in parentheses.

tion. In contrast, many *E. histolytica* isolates have been obtained from asymptomatic cyst passers in Japan (25), so the accurate identification of species is of great significance, both clinically and epidemiologically. Therefore, MAb ED17 may be useful for the specific identification of *E. dispar*.

Identification of *E. dispar* by MAbs may also be possible by the combined use of two MAbs, an *E. histolytica*-specific MAb and a MAb cross-reactive with *E. histolytica* and *E. dispar*. Recently, such MAbs to a 170-kDa amebic lectin were used for antigen capture in serum and feces by enzyme-linked immunosorbent assay (1, 11). We observed that the combined use of MAbs 4G6 and 3F7 was effective for distinguishing both species, although only cultured trophozoites were tested (12a, 26). However, by this method two samples per isolate must be stained separately. An obvious advantage of the double staining achieved by ED17 and 4G6 is that the differentiation of *E. histolytica* and *E. dispar* is possible with just one sample in cases of mixed infection. Other useful procedures available for the specific identification of *E. histolytica* and *E. dispar* are PCR and restriction enzyme digestion of amplified DNA (2, 24, 27, 29). We designed specific primers for the genomic DNA encoding the 30-kDa molecule of *E. histolytica* and the analogous gene of *E. dispar*. After 30 cycles of amplification, *E. histolytica* and *E. dispar* DNAs were specifically detected from cultured trophozoites or cysts (5, 20, 27). By this procedure, we have never observed evidence of mixed infections of *E. histolytica*

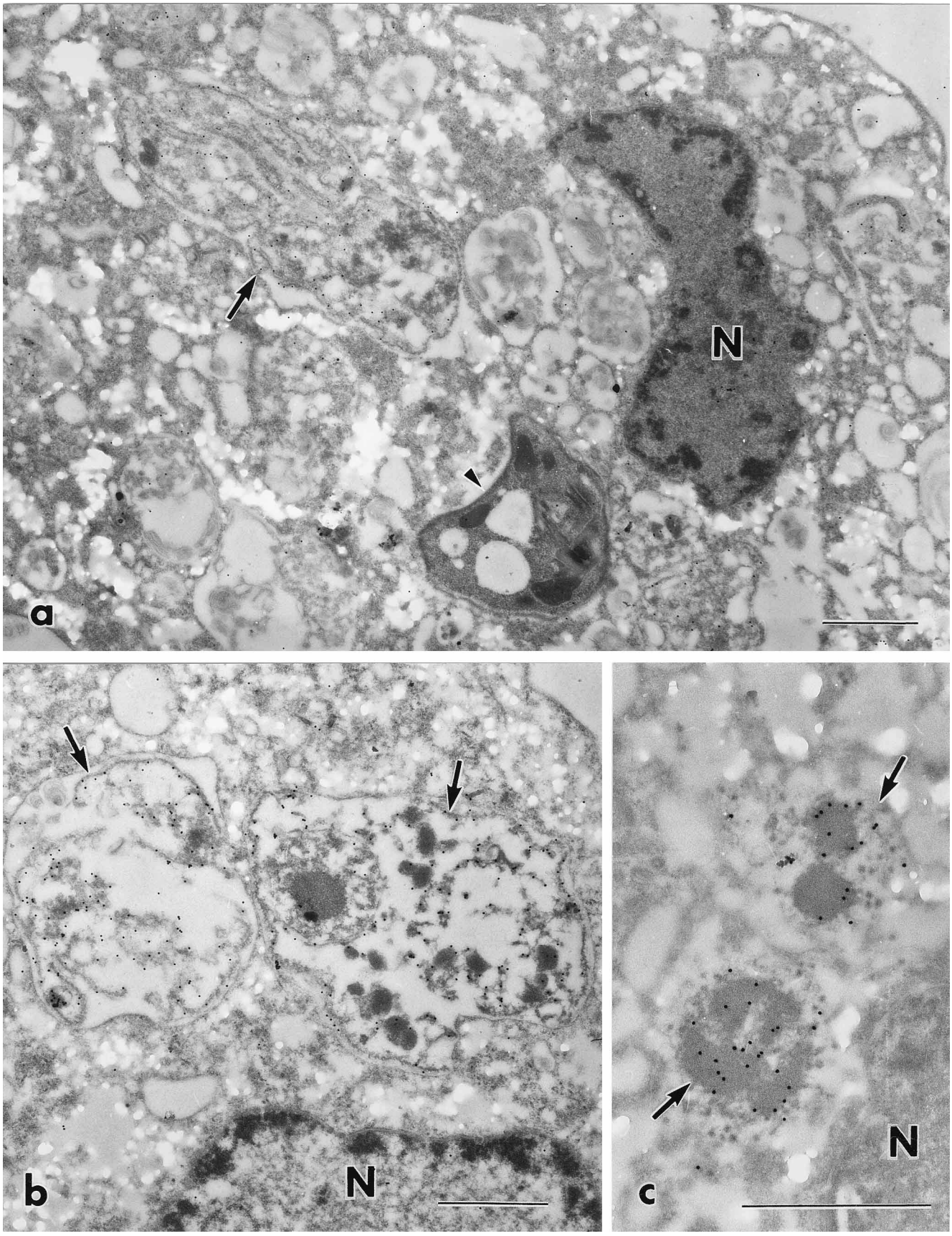

FIG. 2. Localization of the antigen recognized by MAb ED17 by immunoelectron microscopy. Trophozoites of *E. dispar* SAW1734RclAR cultured monoxenically with *C. fasciculata* (a and b) or with *Pseudomonas aeruginosa* (c) were incubated with ED17 (1:20-diluted ascitic fluid), followed by incubation with gold particle-conjugated protein A. N, nucleus of *E. dispar*. Note that gold particles are observed on digested *C. fasciculata* and *P. aeruginosa* (arrows), but not on undigested organisms (arrowhead). Bars, 1 μm.

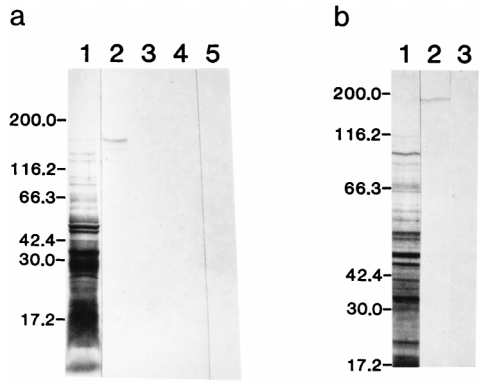

FIG. 3. Western immunoblot analysis of reactivity of MAb ED17 with trophozoites of *E. dispar*. (a) Lanes 1, 2, and 5, *E. dispar* SAW1734RclAR cultured monoxenically with *P. aeruginosa*; lane 3, *P. aeruginosa*; lane 4, *E. histolytica* HM-1:IMSS. Samples were subjected to SDS-PAGE (5 to 20% polyacrylamide gel) and were transferred to polyvinyl difluoride membranes. Protein bands in lane 1 were stained with Coomassie brilliant blue. Lanes 2 to 4 were treated with MAb ED17, followed by horseradish peroxidase-labeled goat anti-mouse IgG and substrate. Lane 5 was treated with normal mouse serum as a control. (b) Effect of periodate treatment on the ED17-reactive epitope. Cultured trophozoites of *E. dispar* were subjected to SDS-PAGE (7.5% polyacrylamide gel), transferred to polyvinyl difluoride membranes, and then treated with periodate. An untreated strip (lane 2) and a strip treated with 10 mM periodate (lane 3) were incubated with ED17, followed by incubation with horseradish peroxidase-labeled goat anti-mouse IgG. Protein bands in lane 1 were stained with Coomassie brilliant blue. In both panels, numbers to the left indicate molecular masses of the markers (in kilodaltons). Molecular weight standards were myosin (200,000), β-galactosidase (116,248), albumin (66,267), aldolase (42,400), carbonic anhydrase (30,000), and myoglobin (17,201).

and *E. dispar*. However, in Mexico, Acuna-Soto et al. (2) demonstrated mixed infections of *E. histolytica* and *E. dispar* in stool samples containing cysts by PCR and subsequent DNA hybridization. If a small number of *E. dispar* organisms are present within a large population of *E. histolytica*, it would be difficult to detect the former by using an *E. histolytica*-specific MAb and a MAb cross-reactive with *E. histolytica* and *E. dispar*. However, even in such cases, the selective staining by *E. histolytica*- and *E. dispar*-specific MAbs may facilitate differentiation.

One of the interesting observations in this study was the localization of the 160-kDa antigen of *E. dispar* recognized by ED17. As shown in Fig. 2, an ultrastructural study of the epitope recognized by ED17 revealed its main localization on *C. fasciculata* and *P. aeruginosa* organisms undergoing intra-amebic digestion. This finding suggests that the 160-kDa antigen may be a substance related to the intracellular digestive processes of the ameba.

The axenic culture of *E. dispar* is difficult because axenization is frequently accompanied by the conversion of the zymodeme pattern from *E. dispar* to *E. histolytica* (3, 15, 16, 18, 32). Axenization of *E. dispar* without zymodeme conversion has been reported for only one strain, by Clark (6). However, the coculturing of *C. fasciculata* or *P. aeruginosa* with *E. dispar* enables the ameba to proliferate in BI-S-33 medium, indicating that some factors essential for growth are supplied by the symbionts. Although the substances have not yet been identified, such factors may be metabolites obtained after digestion of the symbionts. Therefore, localization of the antigen recog-

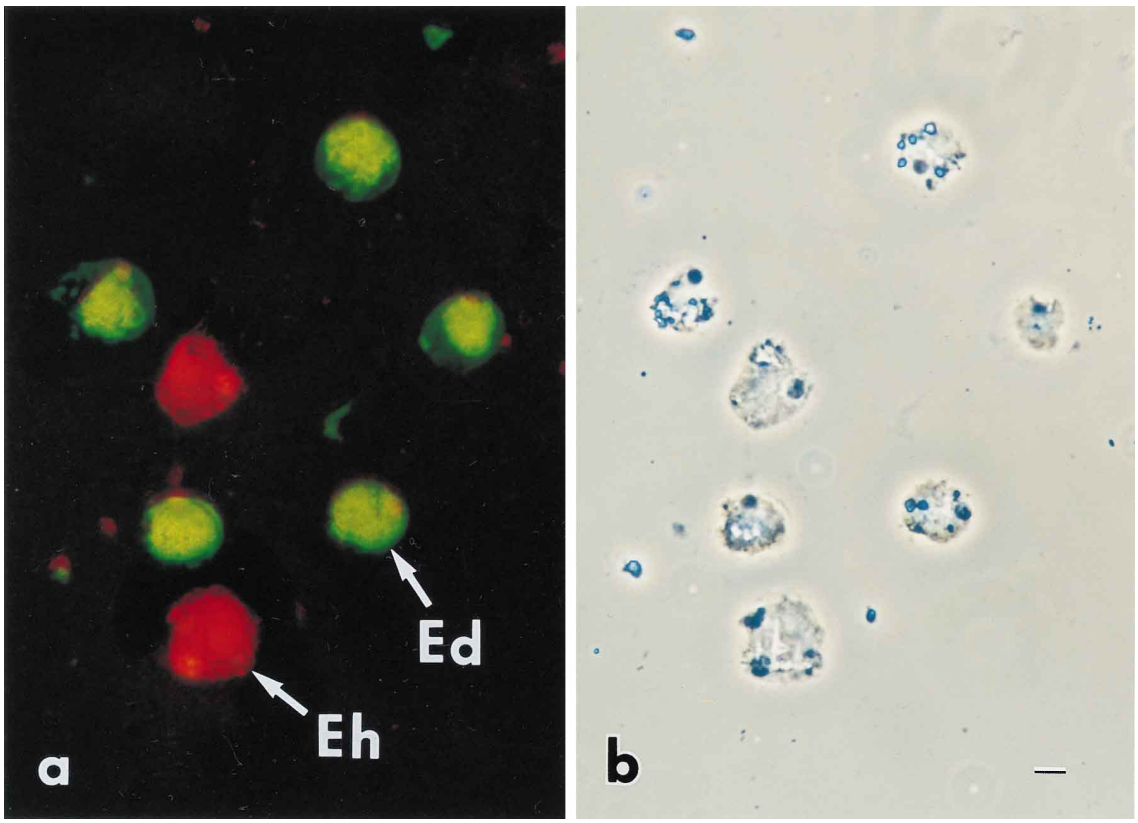

FIG. 4. Immunofluorescence (double staining) (a) and phase-contrast (b) photomicrographs of a mixture of *E. histolytica* and *E. dispar*. Formalin-fixed trophozoites were incubated with MAb 4G6 (IgG1) and MAb ED17 (IgG2a), followed by incubation with a mixture of tetramethylrhodamine isothiocyanate-labeled anti-mouse IgG1 and fluorescein isothiocyanate-labeled anti-mouse IgG2a antibodies. Color film was double exposed by using green and blue excitation filters. Note that *E. histolytica* (Eh) and *E. dispar* (Ed) are clearly differentiated. Bar, 10 μm.

nized by MAb ED17 on digested *C. fasciculata* and *P. aeruginosa* may indicate its relationship to such factors. The molecular analysis of the 160-kDa antigen and clarification of its biological function deserve further study.

ACKNOWLEDGMENTS

We thank W. Stahl for reviewing the manuscript.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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