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A STUDY OF MICROCAPSULE AGGLUTINATION TEST FOR SERODIAGNOSIS OF AMEBIASIS

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Abstract

We established a sensitized microcapsule (MC) agglutination test (MCA-EH) using a chemically stable MC for serological diagnosis of amebiasis, and its ability to detect the antibody was compared with that of the indirect hemagglutination test (IHA).

The antigen-sensitized MC used for the experiment was prepared by adsorption of a sonicate of *Entamoeba histolytica* to the surface of the MC.

The rates of detection of the antibodies in serum specimens from 38 patients with amebiasis and 55 persons excreting the cyst were 97.4% and 20.0%, respectively, by the MCA-EH, which were higher than the 94.7% and 16.4% by the IHA. On the other hand, in 130 serum specimens from healthy subjects serving as the controls, no antibodies were detected by either test.

When reactivity of immunoglobulins was studied by both tests with sera from immunized rabbits, the reaction even with IgM, i.e., the antibody in the early stage of infection was more sensitive in MCA-EH than in IHA.

The MCA-EH gave better results than the conventional IHA test, and it is procedurally simple and the reagents for the test are also stable. For these reasons the MCA-EH test was considered satisfactory for routine examination for amebiasis in the general laboratory.

Introduction

Entamoeba histolytica Schaudin, 1903 is a protozoon of the superclass Rhizopoda, and causes amebic dysentery whose major signs are the symptoms of intestinal amebiasis and amebic liver abscess. The protozoon is a parasite of animals that may be transmitted to man under natural conditions, and is widely distributed throughout the world. According to the World Health Organization[1], there were 480,000,000 persons infected with the microorganism in the world in 1981, and 40,000 to 110,000 persons die from the infection every year. Infection with the protozoon has been observed in homosexual groups at a high incidence in the U.S.[2][3], and it is also increasingly noted as a complication of acquired immune deficiency syndrome, and so on.

Now a days, when there is active interchange of work between Japan and foreign countries, there are many people who are staying for a long time in developing countries centering in the tropics and subtropics where the protozoon is present at a high frequency. The increase in the incidence of amebiasis "imported" to Japan by such returning travelers is also increasingly a problem[4][5].

Amebiasis has been conventionally diagnosed mainly by examination of feces, but detection and identification of the protozoon requires skillful and frequent examinations. Therefore, serological examination is considered to play a major role in diagnosis of the disease.

The serological procedures for amebiasis which are widely used include the gel diffusion precipitation test (GDP), the indirect fluorescent antibody test (IFA), enzyme-linked immunosorbent assay (ELISA) and indirect hemagglutination (IHA)[6]. Among these methods, GDP requires much time

for the results to be obtained, and IFA and ELISA require skillful for evaluation of results and procedure. It is therefore not easy to use these methods in general laboratories. IHA has been shown to be excellent in sensitivity and is not a complicated procedure, leading to its frequent use[7)~9)], but it has the disadvantage of nonspecific agglutination[10)]. In order to avoid this disadvantage, the microcapsule agglutination test (MCA) for serological diagnosis of syphilis and leprospirosis was developed by utilizing chemical stable microcapsules (MC) instead of erythrocytes[11)12)]. Some studies have shown that the MCA induces almost no nonspecific agglutination caused by antibody, in contrast with the conventional IHA, and that it reacted sharply even with the IgM antibody in early infection[11)12)].

We established a sensitive highly reliable MCA for amebiasis (MCA-EH) on the basis of such characteristics of the MC, and used it to study the reactivity of serum samples from patients.

Materials and Methods

1. *Entamoeba histolytica*

The microorganism used was the HM1-IMSS strain grown in axenic culture in BI-S-33 medium (Diamond et al, 1978)[13)].

2. Serum specimens

1) Human serum: Human serum specimens were collected from 38 patients with amebic dysentery (12 with intestinal amebiasis, 22 with amebic liver abscesses and 4 with the combination of intestinal amebiasis and amebic liver abscess), 10 with non-amebic liver abscesses, 10 with bacterial colitis, 183 rheumatoid factor-positive individuals, 93 syphilis-positive individuals, 30 individual infected with other parasites and 55 individuals with asymptomatic cysts of *E. histolytica*. These specimens were collected in the Gastrointestinal Diseases Center, Tokyo Women's Medical College, and other institutions. Serum specimens collected from 103 healthy persons served as the control.

2) Fractionation of serum specimens from immunized rabbits by gel filtration:

E. histolytica antigen at two protein concentrations, 0.25 and 0.5 mg, was subcutaneously inoculated together with complete adjuvant into healthy female rabbits, weighing about 2.5 kg. Serum samples were collected from the aural vein for successive days after the inoculation and were fractionated into IgM and IgG fractions by high-performance liquid chromatography (HPLC). The column used was a G-3000 SW (Tosoh Co., Ltd.). The amount of sample for the fractionation was 0.075 ml and the flow rate was 0.5 ml/min. Phosphate buffered physiological saline, 0.15 M, PBS (pH 7.2) was used as the elution solution. The amount of each fraction was 0.75 ml. The resultant eluate corresponded to a dilution of immune serum diluted to about 1:10. The absorbance of the eluate of each fraction was determined at 280 nm. The absorbance of the eluate of each fraction was determined at 280 nm. The curve of protein absorbance had three main peaks corresponding to fractions of IgM, IgG and albumin, as shown from the left to right in Fig. 4.

3. Preparation of MCA-EH reagents

The following conditions were studied with the MC sensitized at a 320-fold concentration of antigen and unsensitized MC.

1) Dilutions of serum: Dilutions of the test sera were studied with the antigen-sensitized MC described previously and unsensitized MC. The control specimens were 1-0.125% bovine serum albumin (BSA)/PBS and 0.5% BSA/glycine buffered saline (GBS).

2) Antigen solutions: The antigen solutions used were the supernatant fluid from centrifugation at 15,000 rpm, for 30 min and at 3,000 rpm, for 5 min following ultrasonication.

3) Conditions of sensitization with antigen: The amount of antigen solution diluted to the optimum concentration (centrifuged at 3,000 rpm, for 5 min), equivalent to an MC suspension, was added to the MC suspension (as will be described later), and the mixture was allowed to react at 37°C for 30 min or overnight at 4°C.

4) Concentration of MC: In order to study the conditions for obtaining a better MC reaction, MC

suspensions of 1.5, 1.0 and 0.5% were sensitized with the antigen, and MCA-EH was performed with these suspensions.

4. Sensitized MC agglutination reaction test (MCA-EH)

Various conditions of preparation of reagents for MCA-EH were studied, and the following methods were used for the experiment.

1) *E. histolytica* antigen: The antigen was prepared as follows.

When the microorganisms were incubated for 3 days in a screw-capped Erlenmeyer flask containing 120 ml of BI-S-33 medium, they proliferate at the bottom of the flask. After the 80% of the culture solutions were removed with an aspirator, the flask was cooled in ice water for 5 min, with rotation several times to suspend the amebae. The suspension was transferred to centrifuge tubes. After centrifugation at 2,000 rpm, for 5 min, the supernatant fluid was discarded and the pellet was suspended in physiological saline. The same procedure of centrifugation and washing was repeated four times. The number of amebae was adjusted to 10×10^6 per ml by adding physiological saline to the pellet. The suspension was treated by ultrasonication for 5 min at an output of 150 W and a frequency of 20 KHz with an ultrasonic cell crusher, and centrifuged at 3,000 rpm, for 5 min. The supernatant fluid was distributed into small test tubes as the antigen and stored at −80°C.

2) Microcapsule MC particle: After a 10% MC (green; particle size, 0.4 μm) suspension stored at 4°C was centrifuged (2,500 rpm, 10 min) and washed with PBS once, the concentration of MC was adjusted to 0.9% with physiological saline and used for the experiments.

3) Preparation of antigen-sensitized MC suspension: Antigen-sensitized MC suspensions were prepared according to the method of Yamaura et al[14] as shown in Fig. 1.

For sensitization of MC, an amount of the antigen solution diluted to the optimum concentration with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Sigma Chemical Co.) adjusted to 0.2% with PBS equivalent to a 0.9% MC suspension was added to the 0.9% MC suspension, and the mixture was left at 4°C overnight. After the sensitized MC suspension was centrifuged (3,000 rpm, 5 min) and washed with physiological saline three times, the concentration of MC was adjusted to 0.9% with 3% BSA/PBS and stored at 4°C. A suspension of unsensitized MC was prepared similarly by adding an amount of 3% BSA/PBS equivalent to a 0.9% MC suspension to

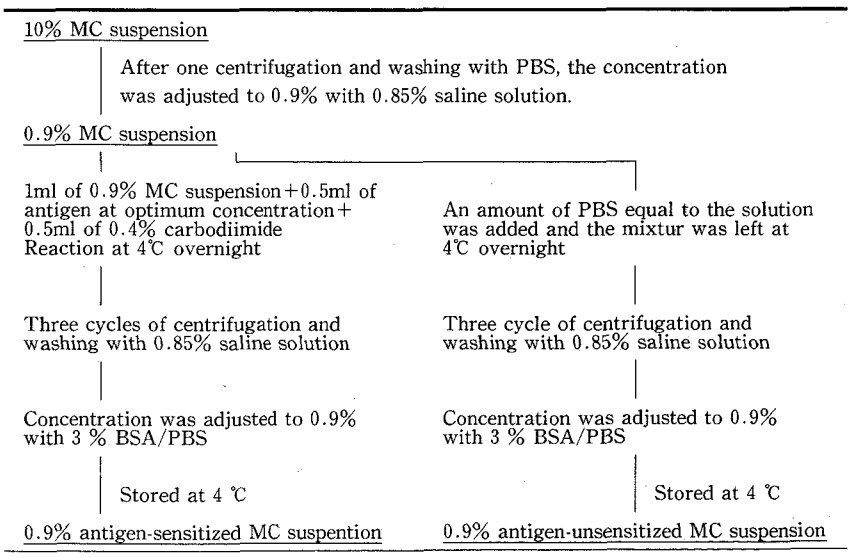

Fig. 1 Preparation of antigen-sensitized and unsensitized MC suspension

the 0.9% MC suspension.

4) Optimum concentration of antigen: In order to obtain an anti-*E. histolytica* antibody titer approximately the same as that obtained by IHA, the optimum concentrations of the antigen for MCA sensitization in 4 serum specimens with IHA antibody titers of <1:80, 1:80, 1:320 and 1:1,280 were determined. The concentrations of antigen ranged from 10- to 1,280-fold.

5) Procedure of MCA-EH: Fig. 2 shows the procedure of MCA-EH. The diluent for the serum i.e., 0.5% BSA/GBS, was distributed with a dropper into U-shaped wells of a microplate: 4 drops (100 μl) in the first well and 1 drop (25 μl) each in the second to the final wells. In the first well, another 25 μl of the test specimen was added with a micropipet. The same number of diluters as that for the test specimens were placed in the first well and rotated 8 to 10 times for dilution. These diluters were moved to the second well, and rotated similary. The same procedure was repeated for the third to the 11th wells. The final well (the 12th well) contained only the diluent. One drop (25 μl) of unsensitized MC suspension was placed in the third well, and then one drop (25 μl) of sensitized MC suspension was placed in each of the fourth to the final well with another dropper. The contents of the microplate were then adequately mixed with a mixer for 10 min, and the microplate was sealed and left standing at room temperature for 90 min. Then the plate was examined grossly for the agglutination pattern of MC in an observation box or on white paper. Positive serum specimens (from immunized rabbits) were treated by the same procedure.

6) Criteria of MCA-EH: Fig. 3 show the criteria of MCA and the pattern of reaction, respectively. The serum dilutions were expressed as the dilution after the addition of MC suspension. The highest final serum dilution giving a MCA pattern of 1+ or more was regarded as the antibody titer, and the antibody titer of >1:80 was determined to be the limit for positivity. In each test an unsensitized MC control was placed in the third well and the diluent control was placed in the final well (the 12th well). Both specimens were confirmed to be negative for sensitization.

4. Reproducibility, time required to obtain results, keeping quality of the reagent, and effect of inactivation of the serum on the results of MCA-EH

Rabbit serum specimens and human serum specimens wit different antibody titers, which ranged from <1:80 to 1:5,120, were used for the experiments.

5. Comparison of the reaction of test sera in MCA-EH and in IHA

The test serum specimens described above were subjected to MCA-EH and IHA, and the reaction of each specimen was studied. The serum specimens from patients without *E. histolytica* who were positive for MCA-EH and IHA (antibody titer >1:80) were assessed by ELISA. Amoebiasis HA-test Kits (Japan Lyophilization Laboratory) were used for IHA, and ELISA was conducted according to the method of Yamaura et al[15].

Cup No.	1	2	3	4	5	6	7	8	9	10	11	12	
Diluent (μl)	100	25	25	25	25	25	25	25	25	25	25	25	
Serum (μl)	25	25	25	25	25	25	25	25	25	25	25	25	25 Discard
Dilution of serum (μl)	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1,280	1 : 2,560	1 : 5,120	1 : 10,240	
Unsensitized MC (μl)			25										
Sensitized MC (μl)				25	25	25	25	25	25	25	25	25	
Final serum dilution			1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1,280	1 : 2,560	1 : 5,120	1 : 10,240	1 : 20,480	
	Unsensitized control			Test									

Fig. 2 Procedure for MCA-EH

Shake the plate for mixing. Incubate at room temperature for 90 min or 18hr and read the reaction patterns.

Results

1. Conditions of preparation of MCA-EH reagents

1) Serum dilution: BSA diluted to 1.0, 0.5, 0.25 and 0.125% with PBS was used as the diluent for the test serum (Table 1). As the concentration of BSA was increased, the MCA-positive reaction became weak. In contrast, although the positive reaction improved as the concentration of BSA was decreased, the negative reaction became poor and the end point became obscure. The optimum concentration of BSA was 0.5%, but in further studies with 100 specimens, nonspecific agglutination of the unsensitized MC was observed in two specimens. Therefore when 0.5% BSA/GBS (BSA was diluted to 0.5% with GBS instead of PBS) was used as the diluent, the nonspecific agglutination in the two specimens disappeared.

2) Antigen solution and conditions of sensitization with antigen: For use as antigen solution, the supernatant fluid obtained by centrifugation at 15,000 rpm, for 30 min following ultrasonication and that fluid obtained by centrifugation at 3,000 rpm, for 5 min were compared. As shown in Table 2, the former solution showed a poor agglutinability because of marked slipping of the agglutinated MC, while the latter one yielded a good result. The method of sensitization of MC with the antigen was studied by incubation at 37°C for

Table 1 Concentrations of BSA as the diluent for the serum of MCA-EH

Concentration of BSA (%)	Dilution of positive control serum 1:							Control diluent
	80	160	320	640	1,280	2,560	5,120	
1	3+	3+	3+	2+	1+	−	−	−
0.5	3+	3+	2+	2+	1+	−	−	−
0.25	2+	2+	2+	2+	1+	−	−	−
0.125	2+	2+	2+	2+	2+	−	−	−

3+, 2+, 1+, − : Pattern of reaction of MCA-EH

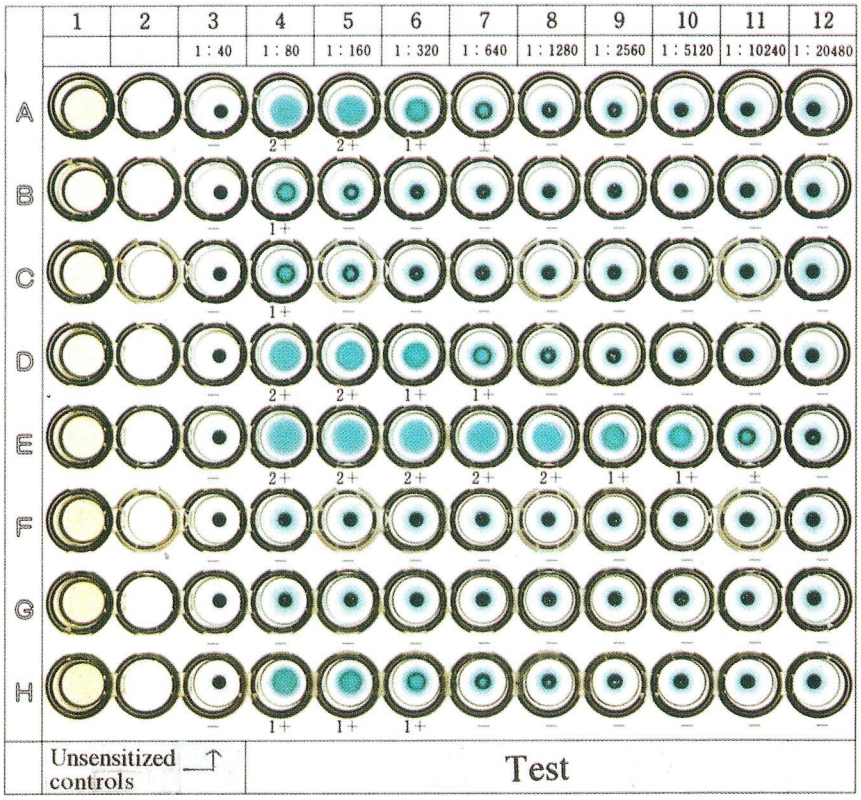

Fig. 3 Pattern of reaction of MCA-EH

Table 2 Agglutinability of antigen in the supernatant obtained by centrifugation at 15,000 rpm, for 30 min and 3,000 rpm, for 5 min

Centri-fugation	Serum	Dilution of positive control serum 1:								Control diluent
		80	160	320	640	1,280	2,560	5,120	10,240	
15,000rpm 30min	A	$3+^{s}$	$3+^{s}$	−	−	−	−	−	−	−
	B	$3+^{s}$	$3+^{s}$	$3+^{s}$	$3+^{s}$	1+	−	−	−	−
	C	−	−	−	−	−	−	−	−	−
3,000rpm 5min	A	2+	+	−	−	−	−	−	−	−
	B	2+	2+	2+	2+	1+	−	−	−	−
	C	−	−	−	−	−	−	−	−	−

3+, 2+, 1+, − : Pattern of reaction of MCA-EH, ^{s}: Slipping of MC.

Table 3 Assessment of the conditions of antigen sensitization at 37℃ for 30 min and 4℃ overnight

Antigen sensiti-zation	Dilution of antigen	Dilution of positive control serum 1:								Control diluent
		80	160	320	640	1,280	2,560	5,120	10,240	
37℃ 30min	10×	2+	2+	2+	2+	2+	2+	2+	2+	2+
	20×	2+	2+	2+	2+	2+	2+	2+	2+	2+
	40×	2+	2+	2+	2+	2+	2+	2+	2+	±
	80×	2+	2+	2+	2+	2+	1+	1+	±	−
	160×	2+	2+	2+	1+	1+	±	−	−	−
	320×	2+	2+	1+	1+	−	−	−	−	−
	640×	2+	1+	−	−	−	−	−	−	−
	1,280×	1+	−	−	−	−	−	−	−	−
4 ℃ overnight	10×	2+	2+	2+	2+	2+	2+	2+	2+	2+
	20×	2+	2+	2+	2+	2+	2+	2+	2+	2+
	40×	2+	2+	2+	2+	2+	2+	2+	2+	2+
	80×	2+	2+	2+	2+	2+	2+	2+	2+	2+
	160×	2+	2+	2+	2+	2+	2+	2+	2+	±
	320×	2+	2+	2+	2+	2+	1+	1+	±	−
	640×	2+	2+	2+	1+	1+	±	−	−	−
	1,280×	2+	2+	1+	1+	−	−	−	−	−

2+, 1+, ±, − : Pattern of reaction of MCA-EH

30 min or at 4°C overnight. The latter procedure yielded an antibody titer about four times higher than the former procedure. Incubation at 4°C overnight was therefore favorable for sensitization (Table 3).

3) Optimum dilution of antigen: The optimum antigen dilution was determined by comparing the results with those obtained by IHA as the control. An antibody titer approximately the same as that found by IHA was obtained at a 1:640 dilution of antigen. The optimum antigen dilution was therefore 1:640.

4) Concentration of MC: The optimum concentration of MC was determined by MCA-EH with 1.5, 1.0 and 0.5% sensitized MC suspensions. With the 1.5% suspension, an negative reaction (failure of MC to form a button-like aggregate) was much more frequent than in the conventional IHA. In regard to the positive reaction pattern, MC had a strong tendency to slip toward the center. With the 1.0% suspension the frequencies of positive and negative reactions were almost the same as those obtained by IHA, and evaluation of the reaction pattern, was easier than that in IHA.

When the 0.5% suspension was used, the positive and negative findings were obscure because of the low concentration of MC. Re-examination showed that the optimum concentration of MC was 0.9%.

2. Reproducibility, time required to obtain results, keeping quality of the reagent and effect of inactivation of the serum on the results of MCA-EH

1) Reproducibility of results: MCA-EH was performed six times on each of 9 rabbit serum specimens. The antibody titers of each serum were incomplete agreement in all six tests.

2) Time required to obtain results: MCA-EH was performed on serum specimens from 11 rabbits, and the time required to obtain results was determined. The plates were sealed after the test was completed and left standing at room temperature for 30, 60, 90, 120 and 180 min, and 18 hr (on the following day), before the results were read. The specimens left for 30 and 60 min yielded well-differentiated positive and negative findings, but it was impossible to determine antibody titers because of obscure and points. Those left for 90, 120, 180 min and 18 hr were easy to read, and showed no difference in antibody titers among the reading times.

3) Stability of MCA-EH reagent after its preparation: The MCA-EH reagent was prepared and stored at 4°C for a fixed period to study its stability (Table 4). MCA-EH was performed on serum specimens from 9 rabbits using the reagent stored at 4°C for 1-180 days, and antibody titers obtained with the reagent in MCA-EH were compared. Even with the reagent stored for 180 days the antibody titers of all of the serum specimens were consistent with those obtained with the reagent used on the day of preparation.

4) Effect of inactivation of the serum: The effect of inactivaiton of the serum (heating at 56°C for 30 min) on results was studied in serum specimens from six humans (Table 5). Inactivation has no effect on the pattern or the antibody titer.

3. Reactivity of the test sera in MCA-EH

1) Qualitative comparison between MCA-EH and IHA of sera from patients and sera from healthy persons: MCA-EH and IHA on serum specimens from patients with amebiasis were compared qualitatively (Table 6). The rate of positivity of specimens from patients with amebic liver abscesses with MCA-EH was 100%, and for specimens from patients with intestinal amebiasis it was 91.7%. The positivity rate for specimens from patients with amebic liver abscesses with IHA was 100%, which was the same as that with MCA-EH, but the rate for intestinal amebiasis with IHA was 83.3%, which was lower than that with MCA-EH. The positivity rate of specimens from the asymptomatic patients excreting *E. histolytica* cysts with MCA-EH was 20.0% (11

Table 4 Assessment of the storage of MCA-EH

Number of days	MCA-EH antibody titers according to serum								
	A	B	C	D	E	F	G	H	I
1	<1 : 80	<1 : 80	1 : 80	1 : 80	1 : 160	1 : 160	1 : 160	1 : 640	1 : 2,560
3	<1 : 80	<1 : 80	1 : 80	1 : 80	1 : 160		1 : 160	1 : 640	
7	<1 : 80	<1 : 80	1 : 80	1 : 80	1 : 160	1 : 160		1 : 640	1 : 2,560
14	<1 : 80	<1 : 80	1 : 80	1 : 80	1 : 160	1 : 160	1 : 160	1 : 640	1 : 2,560
30	<1 : 80	<1 : 80	1 : 80	1 : 80	1 : 160	1 : 160	1 : 160	1 : 640	1 : 2,560
60	<1 : 80	<1 : 80	1 : 80		1 : 160		1 : 80	1 : 640	1 : 2,560
90	<1 : 80	<1 : 80	1 : 80	1 : 80	1 : 160	1 : 160	1 : 160	1 : 640	1 : 2,560
120	<1 : 80	<1 : 80	1 : 80		1 : 160		1 : 160	1 : 640	1 : 2,560
150	<1 : 80	<1 : 80	1 : 80		1 : 160	1 : 160		1 : 640	1 : 2,560
180	<1 : 80	<1 : 80	1 : 80	1 : 80	1 : 160	1 : 160	1 : 160	1 : 640	1 : 2,560

cases), which was lower than in patients with amebiasis, but slightly higher than 16.4% (9 cases) with IHA. Among the specimens from patients with other diseases, 0.9% (3 cases) were positive with MCA-EH and IHA, and the specimens from healthy persons were negative with both tests. As shown in Table 7, there were three rheumatoid factor-positive cases and one syphilis reaction-positive case. The sera No. 82 and 72 were positive with both MCA-EH and IHA, while serum No. 4 was positive with IHA alone and serum No. 142 positive with MCA-EH alone. Three of these serum specimens were positive with ELISA, and all of them showed results similar to those with MCA-EH.

2) MCA-EH antibody titer of the serum specimens from patients with amebiasis and those from persons excreting *E. histolytica* cysts: Of 22 serum specimens from patients with amebic liver abscesses, two (9.1%) had a MCA-EH antibody titer of 1:320, three (13.6%) 1:640, three (13.6%) 1:1,280, six (27.3%) 1:2,560, five (22.7%) 1:5,120, and three (13.6%) ≧1:10,240. Thus, all of the 22 patients had antibody titers of >1:320. Of 12 from patients with intestinal amebiasis, three (25%) had low antibody titers of 1:80 to 1:160. The serum specimens from patients with intestinal amebiasis associated with an amebic liver abscess has antibody titers of 1:640 (1 case) and 1:10,240 (3 cases). In contrast, two serum specimens each from persons positive for *E. histolytica* cysts had titers of 1:80, 1:160 and 1:2,560, one each had titers of 1:320 and 1:1,280, and the titers were 1:640 in three subjects (Table 8).

Table 5 The effect of inactivation of serum on MCA-EH

Inactivation	MCA-HE antibody titers according to serum					
	A	B	C	D	E	F
Before	1 : 80>	1 : 160	1 : 320	1 : 320	1 : 640	1 : 1,280
After	1 : 80>	1 : 160	1 : 320	1 : 320	1 : 640	1 : 1,280

Table 6 Qualitative comparison of MCA-EH and IHA in patients with amebiasis, asymptomatic cyst carriers, nonamebic patients and healthy controls

	No. examined	Agreement			Disagreement			Positive total	
		MCA+ IHA+ (%)	MCA− IHA− (%)	Total (%)	MCA+ IHA− (%)	MCA− IHA+ (%)	Total (%)	MCA (%)	IHA (%)
Amebic liver abscess	22	22	0	22	0	0	0	22 (100)	22 (100)
Intestinal amebiasis	12	10	1	11	1	0	1	11 (91.7)	10 (83.3)
Amebic liver abscess + Intestinal amebiasis	4	4	0	4	0	0	0	4	4
Total	38	36 (94.7)	1 (2.6)	37 (97.4)	1 (2.6)	0	1 (2.6)	37 (97.4)	36 (94.7)
Asymptomatic cyst carriers	55	9 (16.4)	44 (81.5)	53 (96.4)	2 (3.7)	0	2 (3.7)	11 (20.0)	9 (16.4)
Liver disease	10	0	10	10	0	0	0	0	0
Bacterial enteritis	10	0	10	10	0	0	0	0	0
Collagen disease	183	1	180	181	1	1	2	2	2
Positive for syphilis	93	1	92	93	0	0	0	1	1
Parasitic infection*	30	0	30	30	0	0	0	0	0
Total	326	2 (0.6)	322 (98.8)	324 (99.4)	1 (0.3)	1 (0.3)	2 (0.6)	3 (0.9)	3 (0.9)
Healthy controls	130	0	130	130 (100)	0	0	0	0	0

* : *Giardia lamblia, Toxoplasma gondii, Entamoeba coli, Endolimax nana, Ascaris lumbricoides, Trichuris trichiura,* Heterophyidae, *Clonorchis sinensis*

Table 7 Results of ELISA in four serum specimens from patients without amebiasis, for which MCA-EH results differed from IHA results

Serum No.	Disease	MCA-EH antibody titer	IHA antibody titer	ELISA antibody titer
4	Rheumatoid factor -positive	<1 : 80	1 : 640	−
82	Rheumatoid factor -positive	1 : 640	1 : 1,280	+
142	Rheumatoid factor -positive	1 : 640	<1 : 80	+
72	Syphilis-positive	1 : 80	1 : 160	+

Table 8 Results of MCA-EH in patients with amebiasis and asymptomatic cyst carriers

Clinical diagnosis	Number of patients	Number of cases showing MCA-EH titers (%)									Positive cases
		<1 : 80	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1,280	1 : 2,560	1 : 5,120	≧1 : 10,240	≧1 : 80
Amebic liver abscess	22	0	0	0	2 (9.1)	3 (13.6)	3 (13.6)	6 (27.3)	5 (22.7)	3 (13.6)	22 (100)
Intestinal amebiasis	12	1 (8.3)	2 (16.7)	1 (8.3)	2 (16.7)	3 (25.0)	2 (16.7)	1 (8.3)	0	0	11 (91.7)
Amebic liver abscess + Intestinal amebiasis	4	0	0	0	0	1	0	0	0	3	4
Total	38	1 (2.6)	2 (5.3)	1 (2.6)	4 (10.5)	7 (18.4)	5 (13.2)	7 (18.4)	5 (13.2)	6 (15.8)	37 (97.4)
Asymptomatic cyst carriers	55	44 (80.0)	2 (3.6)	2 (3.6)	1 (1.8)	3 (5.5)	1 (1.8)	2 (3.6)	0	0	11 (20.0)

3) Reaction of serum specimens from immunized rabbits in MCA-EH and IHA: For analysis of serum specimens from rabbits immunized with *E. histolytica* in terms of immunoglobulin, rabbit serum specimens taken 7, 14 and 30 days after immunization were fractionated to IgM and IgG fractions, and their reactions in MCA-EH and IHA were studied (Fig. 4).

(1) Reaction of the specimens collected 7 days after immunization: Both IgM and IgG fractions of the serum specimens from rabbit No. 1 had titers of about 1:4 in MCA-EH, but no antibody was detected by IHA. The IgM fraction of the serum specimens from rabbit No. 2 had titers of 1:32 and 1:16 in MCA-EH and IHA, respectively, and the titers of the IgG fraction were 1:16 and 1:4 in MCA-EH and IHA, respectively.

(2) Reaction of the specimens collected 14 days after immunization: The IgM fractions of the serum specimens from rabbits No. 1 and 2 had titers of 1:16 to 1:32 in MCA-EH, and titers of the IgG fractions from these rabbits were 1:64 in MCA-EH. The IgM fraction from rabbit No. 2 had a titer of 1:8 in IHA, but the fraction from rabbit No. 1 contained no detectable IgM. The IgG fractions from rabbits No. 1 and 2 showed increases in titer to 1:64 and 1:32, respectively.

(3) Reaction of the specimens collected 30 days after immunization: The reactivity of the IgM fraction in MCA-EH and IHA decreased with the increase in reactivity of the IgG fraction; the fractions from rabbits No. 1 and 2 showed antibody titers of 1:64 and 1:128, respectively, in IHA, and of 1:128 and 1:256 in MCA-EH.

Discussion

The methods for serological diagnosis of amebiasis which are currently used in Japan are GDP, IFA, ELISA[15)~18)] and IHA[14)]. IHA, which is sensitive and procedurally simple, is excellent as a routine diagnostic test in the general laboratory, but some studies have revealed the disadvantages that the animal-derived erythrocyte itself used as the carrier possesses antigenicity and that there

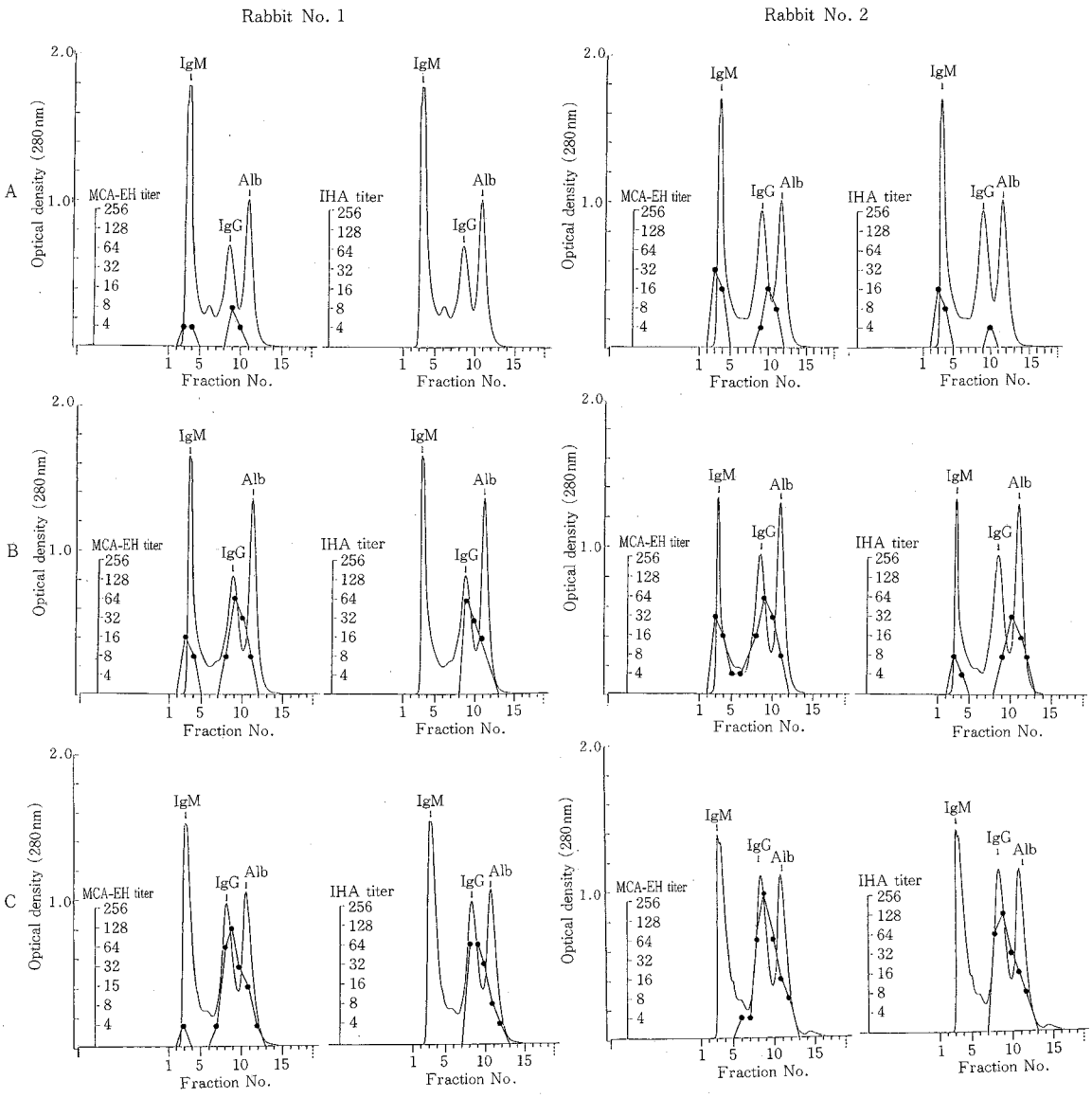

Fig. 4 Antibody activity in MCA-EH and IHA of gel filtrated fraction of rabbit serum collected at 7 days (A), 14 days (B) and 30 days (C) after immunization.
Absorbance:—, Antibody titer: ●—●

are differences among individual animals in the ability of the carrier to adsorb antigen[10)~12)].

To solve these problems, we used chemically stable[11)12)19)] MC without antigenicity as the carrier for the indirect agglutination test and studied the usefulness of the MC as a means of serologically diagnosing amebiasis.

As the diluent for the test serum in MCA, 1% BSA/PBS with 1% sucrose was used by Kobayashi et al[11)], and 1% BSA/PBS by Arimitsu et al[12)]. In our study on 0.125-1% BSA/PBS, 0.5% BSA/PBS yielded good results. Arimitsu et al[12)] found approximately the same tendency. A slight nonspecific reaction was observed with unsensitized MC BSA/GBS at 0.5% was therefore revealed to be effective as a diluent for the test serum.

Kobayashi et al[11] and Arimitsu et al[12] described the preparation of antigen-sensitized MC: Antigen at the optimum concentration is added to MC treated with glutaraldehyde and the mixture is allowed to react at 37°C for 1 hr, and then left at room temperature or 4°C overnight. Since a preliminary experiment revealed that antigen sensitization was inadequate by the conventional method, a method by which good results had been obtained was adopted in the present study; i.e., the antigen diluted at the optimum concentration with 0.2% carbodimeal was added and the mixture was left at 4°C overnight. The MCA-EH reagent prepared in this way was stable and yielded reproducible results even after storage at 4°C for a long time. With IHA using sheep red blood cells the results cannot be obtained for a long time[7][9], but with the present reagent it took only 90 min. Even 18 hr after the first reading, the readings had not changed markedly, and it was also possible to read the test on the following day. Furthermore, inactivation of the test serum did not affect the pattern of the reaction or the readings of the test. Inactivation of the test serum[7][9][10] which is required for IHA with sheep or human red blood cells, was unnecessary. This fact shortened the time required for the test.

The limit for a positive antibody titer with MCA is 1:128->160 according to Arimitsu et al[12], and Kobayashi et al[11] reported that all the serum specimens positive for syphilis had antibody titers of >1:160. The limit, >1:160, in these cases corresponded to >1:320, the dilution used in our MCA-EH. The limit for positivity with the present MCA-EH was considered to be 1:80, as reported by Yamaura et al[14], because all serum specimens from healthy pregnant women had antibody titers of <1:80 and many serum specimens from patients with amebiasis also had antibody titers of ≧1:80.

The rate of IHA positivity for healthy persons is 3% according to Kessel et al[20] and 2.17% according to Agarwal et al[21]. On the other hand, Milgram et al[7], Thompson et al[9] and Patterson et al[10] reported 96%, 100% and 95% for patients with amebic liver abscesses and 82%, 90% and 91% for those with intestianl amebiasis, respectively, as rates of IHA positivity for amebiasis. In our experiment, all serum specimens from healthy persons serving as the negative controls had antibody titers of <1:80 in MCA-EH and IHA, showing that the rate of positivity for both tests was 0%, unlike the results obtained by Kessel et al[20] and Agarwal et al[21]. On the other hand, the serum specimens from patients with amebic liver abscesses were 100% positive in MCA-EH and IHA, as in the results of Thompson et al[9]. The rates were higher than those obtained by Milgram et al[7] and Patterson et al[10], suggesting the effectiveness of MCA-EH and IHA reagents for diagnosis of the disease. With MCA-EH the percentage of positive specimens from patients with intestinal amebiasis was 91.7%, which was lower than that for patients with amebic liver abscesses, but higher than the 82% of Milgram et al[7] and the 83.3% with IHA used in the present study.

It has been shown that there are pathogenic and nonpathogenic strains of *E. histoltica*. Sargeaunt et al[22][23] recently differentiated pathogenic from nonpathogenic strains in studies using isozyme patterns. By using starch gel electrophoresis, they investigated more than 6,000 isolated strains of amebae obtained from various places centering on habitats of *E. histolytica* and found that the pathogenic and non-pathogenic strains show individually specific stable isozyme patterns. In our studies by MCA-EH and IHA on serum specimens from Japanese travelers returning from abroad, who were positive for amebic cysts, the rates of positivity were 20.0 and 16.4%, respectively, which were higher than the 9% for each test obtained by Krupp et al[24], but lower than the 66% obtained by Kessel et al[20]. Thus, results of serological diagnosis using MCA-EH or IHA were not satisfactory. Although the properties the amebic strains detected have not been studied in detail, the low rate of positivity in our present study is believed to be due to differences among the subjects studied and in the pathogenicity of the amebic strains. Serum from the cystpositive sub-

jects frequently had low antibody titers of 1:80 to 1:640. These results were similar to the tendency observed by Krupp[24] showing that the antibody titers were 1:80 to 1:160 in 7% and 1:320 to 1:640 in 2% of the subjects with antibody.

Reactivity of the IgM and IgG fractions in MCA-EH and IHA were studied in immunized rabbits. The reactivity of IgM antibody, which frequently appears in the early stage of infection, in MCA-EH was more than that in IHA, and the increase in titer of the IgG fraction in the early stage with MCA-EH was more marked than that with IHA. The surface of MC, the carrier of MCA-EH, is hydrophobic, while that of red blood cells, the carrier of IHA, is hydrophilic. As in the case of toxoplasmosis[25], therefore, hydrophobic binding of the membrane of amebae to MC, the carrier, is believed to cause the high sensitivity of IgM in MCA-EH.

From the above results, the present MCA-EH reagent was considered effective as a reagent for serological diagnosis in the general laboratory because it gave better results for amebiasis than the conventional IHA and because it has the advantages of procedure simplicity and tolerance to long-term storage.

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マイクロカプセル凝集反応による赤痢アメーバ症の血清学的診断法に関する研究

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赤痢アメーバ症の血清学的診断の目的で，化学的に安定なマイクロカプセル（MC）を用いた感作MC凝集反応（MCA-EH）を確立し，抗体の検出状況について，間接赤血球凝集反応（IHA）による成績と比較検討した．

実験に使用した抗原感作MCは，赤痢アメーバ虫体の超音波処理成分を，MCの表面に吸着させて作製したものである．

MCA-EHの赤痢アメーバ患者38例およびシスト排泄者55例の血清に対する抗体検出率は，各々97.4，20.0%で，IHAによる各々94.7，16.4%に比べて高値であった．一方，対照として用いた健康人血清130例では，MCA-EHおよびIHAによる抗体検出率は共に0%であった．

免疫家兎血清を使用して，両法による免疫グロブリンクラスでの反応性を調べた所，MCA-EHは感染初期抗体であるIgMに対してもIHAより鋭敏に反応することが判明した．

本MCA-EHは，従来のIHAより成績が良好で，操作が簡便かつ，試薬も安定であるなどの利点を有するので，一般検査室における赤痢アメーバ症の日常検査用として有効と思われた．