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Application of the Indirect Hemagglutination Test Using Glutaraldehyde-fixed Chicken Red Blood Cells to Serological Diagnosis of Amebiasis

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Abstract

An indirect hemagglutination test (IHA), using glutaraldehyde-fixed chicken red blood cells (GFC-IHA) has proved to be technically simple, time-saving and reliable for the serological diagnosis of amebiasis. The GFC-IHA was performed in 86 cases of human amebiasis. It was positive in an average of 95.3% of cases (100% of 41 cases of amebic liver abscess, 90.2% of 41 cases of intestinal amebiasis and 100% of 4 cases of combined hepatic and intestinal amebiasis). However, it was positive in only 8.0% of 50 asymptomatic cyst carriers. The GFC-IHA was positive in 0% of 200 healthy controls and in 0.9% of 549 patients with other diseases. Enzyme-linked immunosorbent assay showed similar results in the same groups, namely, 96.0% (100% of 25 cases of amebic liver abscess, 90.5% of 21 cases of intestinal amebiasis, and 100% of 4 cases of combined hepatic and intestinal amebiasis), 10%, 0% and 0.7%, respectively. GFC-IHA titers before and after treatment were determined in 6 patients with amebiasis. Two patients became sero-negative 6 months after treatment. Three cases showed a 4-fold or 8-fold decline in titer at 12 months and one case showed no change in antibody level.

It is suggested that GFC-IHA is a useful method for routine work to the serological diagnosis of amebiasis in general laboratories.

Key words: Amebiasis, *Entamoeba histolytica,* indirect hemagglutination test, chicken red blood cells, glutaraldehyde, IHA

Introduction

Amebiasis has recently been recognized by medical scientists as an important disease because of its widespread incidence in the world, especially in the developing countries (WHO, 1985). Nowadays, the disease is known to be a complication of sexually-transmitted diseases (Phillips *et al.*, 1981; McMillan *et al.*, 1984), as well as an imported parasitic infection in Japan (Yamaura *et al.*, 1981; Yamaura *et al.*, 1983).

Many reliable serodiagnostic tests for amebiasis have been reported, for example, the gel diffusion precipitation test (GDP), the indirect immunofluorescent antibody test (IFA), the enzyme-linked immunosorbent assay (ELISA) and the indirect hemagglutination test (IHA) (Krutschmer, 1986).

IHA has been commonly used in the United States for its high sensitivity in the diagnosis of amebiasis, but almost never in Japan. In IHA, fresh sheep and human red blood cells (RBC) have conventionally been used (Milgram *et al.*, 1966; Thompson *et al.*, 1968; Krupp, 1969) but these made IHA a technically complex method and false-positive results were sometimes a

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problem (Patterson *et al.*, 1980). When IHA was carried out in our laboratories for the detection of amebiasis, the same test using glutaraldehyde-fixed chicken RBC (GFC-IHA) proved to be technically simple, time-saving and reliable. This test was especially good in screening for amebic liver abscess, in comparison with the previously described IHA tests (Yamaura and Shirasaka, 1988; Yamaura *et al.*, 1988). In view of the recent increase in amebiasis in Japan, this new IHA method will be useful for routine work in general laboratories.

This report presents the technique of GFC-IHA applied in the serological diagnosis of intestinal and extraintestinal amebiasis, and the results obtained.

Materials and Methods

Sera: Human sera were collected from subjects attending the Tokyo Women's Medical College, Osaka City University Medical School and Fujita-Gakuen Health University School of Medicine. Eighty-six amebiasis cases awaiting GFC-IHA were first diagnosed by fecal examination and GDP by the method described by Takeuchi and Kobayashi (1983). Of the total cases, 41 had amebic liver abscess, 41 had intestinal amebiasis, 4 had both hepatic and intestinal amebiasis and 50 were asymptomatic cyst carriers. In addition, 549 cases of nonamebic diseases were diagnosed with GFC-IHA. Of these cases, 30 involved liver diseases, 30 involved bacterial enteritis, 250 involved collagen diseases, 159 involved syphilis, and 80 involved other parasitic diseases. Finally, sera from 200 healthy pregnant women were used as normal controls.

GFC-IHA titers before and 6 or 12 months after treatment were determined in 6 patients (4 with amebic liver abscess and 2 with intestinal amebiasis). All sera were stored at −20°C or −70°C until use.

Antigen: The amebic antigen was prepared from *Entamoeba histolytica* (HM-1: IMSS strain) axenically grown in BI-S-33 medium (Diamond *et al.*, 1978) by the method described by Kessel *et al.* (1965) and Takeuchi *et al.* (1977) with some modification. In brief, after cultivating at 35.5°C for 3 days, amebae were harvested and washed by centrifugation 4 times with 0.85% saline. The concentrated amebae were resuspended with 0.85% saline to yield 1×10^7 amebae/ml and disintegrated by sonification at 20 KC for 2 mins. The antigen was extracted at 4°C for 2 days during continuous stirring. After the extract was centrifuged at $20,000 \times g$ for 1 hr, the resulting supernatant was filtered through a Millipore membrane of 0.45 μm porosity, and was kept in small aliquots at −70°C until use.

GFC-IHA: The GFC-IHA used antigen-sensitized chicken RBC, unsensitized chicken RBC, positive control serum (antibody titer 1:1280 determined by IHA) and diluent (0.25% autoclaved normal rabbit serum). The GFC-IHA was prepared as described previously (Yamaura *et al.*, 1988) with some modification, in which 3 mg/dl of tannic acid for treatment of RBC was substituted for 1.5 mg/dl of the same solution (Table 1). Each vial of the GFC-IHA except for the diluent was lyophilized and stored at 2°C to 10°C. Each reagent was reconstituted with diluent to the original volume immediately before use. The expiration date of the GFC-IHA was 2 years after preparation. Reconstituted reagents should be used within 2 days when stored at 4°C, or within 90 days when at −80°C.

IHA: IHA was carried out using a modification of the procedure described by Cox *et al.* (1969). In brief, 25 μl of serially diluted serum in the diluent of GFC-IHA was dispensed into the well of a U-shaped microplate. Inactivation of the sera prior to use is not necessary (Yamaura *et al.*, 1988). Serial dilution of the control serum was made as well. To each well, 75 μl of a suspension of sensitized RBC was then added. The results were read after incubation for 90 min. at room temperature. The incubation time could be extended without perceptible difference in the agglutination of cells. The end-point of each titration was set as the highest dilution of serum still causing visible agglutination of the cells. Titers greater than the test serum dilution of 1:80 were regarded as positive. The reactions of each sample serum (1:40 diluent) and unsensitized RBC were confirmed negative.

To confirm the results of GFC-IHA, ELISA

Table 1. Antigen-sensitized chicken RBC and tanned RBC technique

1% Glutaraldehyde fixed chicken cells
| Wash by centrifugation (3,000 rpm, 5 minutes) five times with 0.85% saline solution.
Resuspend the cells to 4% in phosphate-buffered saline (PBS)-pH 7.2
|
2 ml of 4% cell suspension + 2 ml of tannic acid solution (3 mg/dl in PBS-pH 7.2)
| Agitate gently at 37°C for 30 minutes.
| Wash by centrifugation five times with 0.85% saline solution.
2 ml of 4% tanned cells in PBS-pH 6.4 + 2 ml of antigen diluted in PBS-pH 6.4
| Agitate gently at 37°C for 30 minutes.
| Wash by centrifugation three times with PBS-pH 7.2.
Resuspend the cells in 2 ml of 1% normal rabbit serum (NRS)
| Stand at 4°C for 1~2 weeks.
| Wash by centrifugation with PBS-pH 7.2.
Resuspend the cells to 0.4% in 0.25% autoclaved NRS and lyophilize

was also performed by the method described by Takeuchi *et al.* (1988), on patients with sera from 50 amebiasis, on sera from 50 asymptomatic cyst carriers, the sera from all healthy pregnant women tested and patients with other diseases.

Results

Comparative results between GFC-IHA and ELISA are shown in Table 2. An GFC-IHA was performed on sera from 86 amebiasis patients

Table 2. Comparative results between GFC-IHA and ELISA in cases of amebiasis, asymptomatic cyst carriers, nonamebic patients and healthy controls

Clinical diagnosis	Number of patients	GFC-IHA		ELISA	
		Examined	Positive (%)	Examined	Positive (%)
Amebic liver abscess	41	41	41 (100)	25	25 (100)
Intestinal amebiasis	41	41	37 (90.2)	21	19 (90.5)
Amebic liver abscess + Intestinal amebiasis	4	4	4	4	4
Total	86	86	82 (95.3)	50	48 (96.0)
Asymptomatic cyst carrier	50	50	4 (8.0)	50	5 (10.0)
Liver disease	30	30	0	30	0
Bacterial enteritis	30	30	0	30	0
Collagen disease	250	250	1 (0.4)	250	0
Positive for syphilis	159	159	4 (2.5)	159	4 (2.5)
*Parasitic infection	80	80	0	80	0
Total	549	549	5 (0.9)	549	4 (0.7)
Healthy controls (Pregnant women)	200	200	0	200	0

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

resulting in a 95.3% (82/86) incidence. In sera from 41 patients with amebic liver abscesses, 100% were positive, in sera from 41 patients with intestinal amebiasis, 90.2% (37/41) were positive and in sera from 4 patients with combined hepatic and intestinal amebiasis, 100% were positive. Antibodies were detected in only 8.0% of sera from 50 asymptomatic cyst carriers and all sera from healthy pregnant women were negative. An ELISA showed similar results for the same groups, 96.0% (100% of 25 cases of amebic liver abscess, 90.5% of 21 cases of intestinal amebiasis and 100% of 4 cases of both hepatic and intestinal amebiasis), 10% and 0%, were respectively. Five hundred and forty-nine serum samples from other disease patients were positive in only 0.9% and only one serum from a collagen disease patient and 4 syphilis-positive serum samples were GFC-IHA positive (1:80). An ELISA was conducted on all these samples and it was found that 0.7% of samples from patient with other diseases was positive. The results of GFC-IHA and ELISA were in agreement except for one specimen from a collagen disease patient which was found positive by GFC-IHA only; agreement rate: 99.8%.

The distributions of GFC-IHA antibody titers of amebiasis and asymptomatic cyst carriers are given in Table 3. While all cases showed-titer of $\geqq$1:320 (1:320 $\sim$ $\geqq$1:5120) and high titers of $\geqq$1:5120 were observed in 46.3%, of serum samples in cases of amebic liver abscess, 6 cases of intestinal amebiasis (14.6%) demonstrated low titers of 1:80 $\sim$ 1:160. In 4 asymptomatic cyst carriers, the antibody titer was 1:80, 1:160, 1:320 and 1:640 respectively. In 4 positive cases of other diseases, all showed IHA titers of 1:80.

The changes in antibody levels before and after treatment are shown in Table 4. Of 6 patients, 2 (cases 1 and 5) became sero-negative

Table 4. Changes in GFC-IHA titer before and after treatment for amebiasis

Case	GFC-IHA titer		
	Before treatment	6 months after treatment	12 months after treatment
1	1:640	<1:80	N.D.
2	1:1280	1:320	1:160
3	1:5120	1:5120	1:5120
4	1:20480	1:10240	1:5120
5	1:320	<1:80	N.D.
6	1:5120	1:2560	1:1280

N.D.: Not done
Cases 1–4 had amebic liver abscess, cases 5–6 had intestinal amebiasis.

Table 3. Results of GFC-IHA in cases of amebiasis and asymptomatic cyst carriers

Clinical diagnosis	Number of patients	Number of cases showing GFC-IHA titers (%)								Positive cases
		<1:80	1:80	1:160	1:320	1:640	1:1280	1:2560	≧1:5120	≧1:80
Amebic liver abscess	41	0	0	0	3 (7.3)	5 (12.2)	7 (17.1)	7 (17.1)	19 (46.3)	41 (100)
Intestinal amebiasis	41	4 (9.8)	2 (4.9)	4 (9.8)	5 (12.2)	2 (4.9)	5 (12.2)	5 (12.2)	14 (34.1)	37 (90.2)
Amebic liver abscess + Intestinal amebiasis	4	0	0	0	0	2	1	1	0	4
Total	86	4 (4.7)	2 (2.3)	4 (4.7)	8 (9.3)	9 (10.5)	13 (15.1)	13 (15.1)	33 (38.4)	82 (95.3)
Asymptomatic cyst carriers	50	46 (92.0)	1 (2.0)	1 (2.0)	1 (2.0)	1 (2.0)	0	0	0	4 (8.0)

6 months after treatment, 3 cases (cases 2, 4 and 6) showed a 4-fold or 8-fold decline in IHA titer at 12 months and 1 case (case 3) showed no change in antibody level.

Discussion

Fecal examination is usually used in diagnosing amebiasis, but is not suitable for the diagnosis of chronic or parenteral infection.

Presently, GDP, IFA, and ELISA are major methods for the serological diagnosis of amebiasis used in Japan (Takeuchi *et al.*, 1985, 1988). However, the GDP cannot measure antibodies quantitatively and is a time-consuming test. The IFA and ELISA require skillful techniques of testing and interpretation of results, and are difficult to use in laboratories in general.

In IHA, sheep and human RBC have conventionally been used. The previously described IHA has been commonly used for its high sensitivity in the diagnosis of amebiasis, but these made IHA technically complex and false-positive results were sometimes a problem (Prakash *et al.*, 1969; Krupp, 1970; Patterson *et al.*, 1980). We studied the applicability of the GFC-IHA using glutaraldehyde-fixed chicken RBC, to the serological diagnosis of amebiasis.

Kessel *et al.* (1965) reported the results of IHA to be false-positive results in 3% of 101 uninfected controls but complement fixation tests gave negative results in all cases and Agarwal *et al.* (1981) found false-positive results in 2.17% of 46 healthy controls. Patterson *et al.* (1980) reported the IHA to be more sensitive than GDP and fecal examination. The GDP is known for its small false-positive and false-negative rates in comparison to IHA and fecal examination. However this GFC-IHA was false-positive in 0% of 200 healthy pregnant woman screened as normal controls. Thus some of our IHA results may have been falsely low.

In IHA using chicken RBC, absorption of the normal serum was not necessary owing to weak heterophilic antigenit and inactivation of the sera prior to use is not necessary. (Oniki and Kurakazu, 1980; Yamaura *et al.*, 1988). This GFC-IHA method proved to be technically simple.

Milgram *et al.* (1966), Thompson *et al.* (1968) and Patterson *et al.* (1980) reported results of the IHA to be positive in 96%, 100% and 95% respectively in three trials on sera from amebic liver abscess patients and 82%, 90% and 91% respectively in three trials on sera from intestinal amebiasis patients. The positivity rates in our trial were about the same as those of Thompson *et al.* (1968) and were higher than those of Milgram *et al.* (1966) and Patterson *et al.* (1980). On the other hand, the rate was only 8.0% for sera from asymptomatic cyst carriers. This positive result approximately agrees with those of Krupp (1965) and Milgram *et al.* (1966) which were both 9% but is much lower result than the of Kessel *et al.* (1965) which was 66%. A few years ago Sargeaunt (1987) reported that by using isozyme patterns Entamoeba can be separated into pathogenic and non-pathogenic strains. The characteristics of strains detected were not examined in that study. However, the low positivity rate may be explained by different pathogenecity among strains. Patterson *et al.* (1980) reported that the magnitude of IHA titer does not correlate with the severity of illness. For example, an infant with amebic colitis and liver abscess proven at postmortem examination had titers of 1:128. With regards the distribution of GFC-IHA titers in amebiasis, all the cases showed $\geqq$1:320 (1:320 $\sim$ $\geqq$1:5120) and high titers of $\geqq$1:5120 were observed in 46.3% of sera from patients with amebic liver abscess, but 6 patients with intestinal amebiasis (14.6%) had low titers of 1:80 $\sim$ 1:160. Among the 549 serum samples from patient with other diseases 5 (0.9%) were positive in GFC-IHA (1:80), but 1 (0.2%) of these was found negative by ELISA. Accordingly, when a low titer such as 1:80 is observed, it becomes necessary to refer to evaluation results of an other test such as ELISA and IFA.

IHA titers before and 6 or 12 months after treatment were determined in 6 patients who cured clinically or turned parasitic negative. Krupp (1970) reported that the IHA antibody titer in amebiasis persisted for at least 6 months after treatment. Juniper *et al.* (1972) found that

the IHA was somewhat more sensitive and the result tended to remain positive longer after cure and that the results of IHA often remained positive for 6 to 12 months, and occasionally for 1 to 3 years. Patterson *et al.* (1980) reported that 9 patients were followed up from the onset of illness for 36 months. All except 2 had negative results in GDP at the end of 6 months. However, the shortest duration of positivity in IHA was 36 months in 1 patient, and the remainder were positive at the end of the follow-up period. However in our cases, 2 of 6 became sero-negative with in 6 months after treatment, 3 cases showed a 4-fold or 8-fold decline in titer at 12 months and 1 case showed no change in antibody level.

The GFC-IHA has proved to be reliable, technically-simple, the reagents are stable for a long period. Therefore, the GFC-IHA could be a superior method for routine work to the serological diagnosis of amebiasis in general laboratories.

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