Seroepidemiology of Anti-Entamoeba Histolytica Antibody by Enzyme-linked Immunosorbent Assay in the Greater Tokyo Area

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An enzyme-linked immunosorbent assay (ELISA) for detecting antibody against *Entamoeba histolytica* was used in a survey to determine the prevalence of *E. histolytica* infection in the greater Tokyo area. Serum samples were collected from 1,869 randomly chosen, symptom-free volunteers (1,645 males and 224 females). Seventeen individuals (0.91%), 14 males and three females, had ELISA values that were interpreted as positive. The calculated histogram of the anti-*E. histolytica* IgG values, after logarithmic transformation of the original skewed values, revealed a normal (Gaussian) distribution. It was also found that the results of the ELISA were closely correlated with those obtained by an indirect immunofluorescent assay (IFA).

(Key words: Entamoeba histolytica, Epidemiology, Enzyme-linked immunosorbent assay)

INTRODUCTION

In Japan, amebic dysentery is a communicable disease that must be reported to the Ministry of Health and Welfare. Numerous cases of amebic dysentery occurred immediately after World War II, but only 10 to 20 patients a year were reported in the 1970s. However, the incidence has recently been increasing, with 98 cases reported in 1989 (3). Of these, domestically acquired infections outnumbered the imported cases by more than two to one (13). Exposure to the parasite results in either asymptomatic infection or symptomatic disease (amebiasis). Most infected individuals are asymptomatic carriers of the parasite since as a commensal *E. histolytica* induces no signs or symptoms. However, as a pathogen, it is the cause of amebiasis which varies in overall prevalence (11). Because an inapparent infection can change into a symptomatic disease, it is important to estimate the prevalence of amebic infection in populations living within circumscribed areas. We measured anti-*E. histolytica* IgG by an enzyme-linked immunosorbent assay (ELISA) to determine the incidence of the specific antibody in asymptomatic individuals living in a densely populated urban area of Japan.

MATERIALS AND METHODS

Sera

Serum samples from 1,869 volunteers (1,645 males and 224 females) were collected and stored at \(-20^\circ\text{C}\) until used. The volunteers lived in the greater Tokyo area, including Yokohama and Kawasaki City, and underwent health screening and were judged to be healthy by the results of a blood chemistry examination. The ages ranged from 19 to 66.

Antigen for ELISA

*E. histolytica* (HK-9 strain), maintained axenically in Diamond's medium (2), was used. Cultured organisms were harvested by centrifugation. The supernatant was removed by aspiration, and the sedimented organisms were washed three times in 0.01 M phosphate buffered saline (PBS), pH 7.2. The sedimented parasites were resuspended in 1 ml of 0.01
M carbonate buffer, pH 9.6, and ruptured by sonication. The sonicate was centrifuged at 10,000 g for 20 min at 4°C and the supernatant used as antigen. To test cross-reactivity, an antigen of *Giardia lamblia*, prepared by a similar procedure, was used. The protein concentrations of the two antigenic preparations were determined by the method of Lowry et al. (8). The final protein concentration of both antigenic solutions was adjusted to 10 μg/ml in the carbonate buffer.

ELISA

ELISA was performed as previously described (15). An 0.1 ml aliquot of antigen solution was applied to each well of an ELISA plate containing 96 flat-bottomed wells (Sumitomo Bakelite, Tokyo), and incubated for 2 hr at 37°C. The plate was then washed 3 times with 0.01 M PBS supplemented with 0.05% Tween 20 (PBS-T). Test sera were diluted 1:400 with PBS-T containing 1% bovine serum albumin (BSA/T), and 0.1 ml aliquots added to the wells. After incubation for 1 hr at 37°C, the plate was again washed with PBS. Goat anti-human IgG serum (heavy and light chain specific), conjugated with horseradish peroxidase (Cappel), was diluted 1:1,000 in BSA-T and 0.1 ml was added to each well. The plate was incubated for another 1 hr at 37°C, and then washed 3 times with PBS-T. Next, 0.1 ml of a 0.03% 2,2'-azinobis (3-ethylbenzthiazoline sulfonic acid) diaminonitramine salt (Sigma), containing 0.005% hydrogen peroxide, was added to each well. After a final incubation of 1 hr at room temperature, 25 μl of a 1.25% sodium fluoride solution was added to each well to stop the reaction. The results were expressed as optical density at 405 nm (OD405). All determinations were performed in triplicate.

Indirect immunofluorescent assay (IFA)

To estimate the sensitivity of ELISA, IFA was used to screen 89 individuals showing the highest values in ELISA. Antigen for IFA was prepared as follows: cultured amebas were collected, washed with PBS, fixed in 3% formalin in PBS for 30 min at 4°C, and then washed with PBS three times. The fixed amebas were resuspended in PBS at a concentration of 4 x 10^5 organisms/ml. Aliquots of 5 μl of the amebic suspension were added to the 12 wells (4 mm in diameter) of a heavy teflon-coated slide (Bokusui Brown, Tokyo), dried at room temperature, and stored at −70°C until used. All procedures were carried out at room temperature. To prevent non-specific reactions, 25 μl of PBS containing 1% BSA (BSA/PBS) was added to each well and incubated for 15 min at room temperature. After washing three times in PBS, 25 μl of test serum diluted 1:4, 16, 64, 256, 1,024 and 4,096 with 1% BSA/PBS was placed on the glass slides and incubated for 30 min in a moist chamber. After washing, FITC-conjugated goat anti-human IgG serum (MBL Co, Nagoya), diluted 1:50, was added and the slides were incubated for another 30 min. After a final washing, the test area was covered with a 50% glycerin solution. Immunofluorescence was detected by fluorescent microscopy, and compared with the negative controls. The final dilutions resulting in fluorescence were recorded as the IFA titers.

RESULTS

As shown in Fig. 1, distribution of the 1,869 ELISA values was asymmetric, with a decided shift to the left. When these data were plotted on a logarithmic normal distribution graph, a good correlation to a logarithmic normal line was observed (r = 0.993) (Fig. 2). According to the logarithmic analysis, the distribution of anti-*E. histolytica* IgG was closely correlated with the calculated curve (Fig. 3). The logarithmic mean and one standard deviation (SD) were −1.287 (OD405 = 0.052) and 0.293, respectively. The 95% confidence interval (mean ±1.96SD) ranged from 0.014 to 0.194 at OD405, and the 99% confidence interval (mean ±2.58SD) was from 0.009 to 0.294 at OD405. Accordingly, 44 (2.4%) of the 1,869 individuals were above the upper limit of the 95% confidence interval, and 17 (0.91%) greater than the 99% confidence interval.

To evaluate cross-reactivity with other intestinal protozoa, anti-*G. lamblia* IgG was also measured by ELISA in the 100 individuals showing the highest anti-*E. histolytica* IgG. Cross-reactivity was not observed (r = 0.006, p > 0.05) (Fig. 4).

To ascertain the reliability of the ELISA, 89 serum samples with the highest values were examined by IFA, and titers ranging from 4 to 64 were recorded. The correlation between the
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**Fig. 1** Distribution of ELISA values (anti-*E. histolytica* IgG) obtained from the sera of 1,869 symptom-free volunteers.

**Fig. 2** The cumulative frequency rate and coefficient line after conversion of the ELISA values (anti-*E. histolytica* IgG) into logarithmic values. Correlation coefficient: $r = 0.993$. 
ELISA values and IFA titers was estimated with a 99% confidence interval. The ELISA values were divided into two groups, one higher than 0.294 at OD405 and the other lower. Of 17 sera in the higher group, 16 (94%) had an IFA titer of 64, whereas 66 of the 72 sera in the lower group (92%) had IFA titers of less than 16 (Fig. 5). Therefore, the results obtained by both serologic tests showed good agreement, even in the group with the high ELISA values.

**DISCUSSION**

In a 1965 survey by fecal examination of the greater Tokyo area, Hori (4) estimated that the incidence of *E. histolytica* cyst passers was 0.8%. Since no other large scale surveys have been performed since then, the present prevalence of asymptomatic amebiasis in the greater Tokyo area is uncertain. The number of cases of amebic dysentery has been increasing...
within recent years based on official reports (3). Because the incidence of invasive amebiasis in homosexual men is high, amebiasis is also considered a sexually transmitted disease (1).

The majority of individuals infected with *E. histolytica* remain symptom-free. Since asymptomatic cyst passers are the major source of infection, it is very important to detect and treat them to prevent the infection from spreading. In contrast, patients with amebic dysentery excrete mainly trophozoites in their stools, and it is unlikely they are an important source of infection. Large scale surveys are therefore needed to estimate the prevalence but for such surveys, fecal examination rarely is the diagnostic method of choice. There are some inherent limitations in microscopic examinations such as the requirement for skilled microscopists and the necessity for repeated examinations because of periodicity and variation in the number of parasites discharged in the stool. To overcome these difficulties, many immunological methods have been applied to diagnose amebic infection and amebiasis including the gel diffusion precipitation test (9), latex agglutination assay (10), indirect hemagglutination (7), complement fixation test (7), immunofluorescence assay (5) and ELISA (16). We chose ELISA and IFA for the present survey; ELISA for its proven sensitivity and IFA as a confirmatory procedure.

The anti-*E. histolytica* IgG of 1,869 asymptomatic volunteers was determined. The distribution curve we obtained approximated those of IgE, alkaline phosphatase, glutamate oxaloacetic transaminase and glutamate pyruvate transaminase in healthy people (6). The means ± 2SD (or 3SD) and a 95% (or 99%) confidence interval have been frequently used as cut-off values for negative controls (12, 14). However, it is meaningless to judge individuals as serologically positive (infected) solely from their serologic values because the distribution of meaningful values is non-normal. As our skewed distribution curve demonstrated with its shift to the left (Fig. 1), the true cut-off value was lower than that calculated. As a result, the positive rate increased with a concomitant increase in the number of false-positives, even if the readings were in the upper limits of the 95 and 99% confidence interval. In a non-endemic area such as Japan, it is doubtful that the cut-off value can be determined simply by a 95% confidence interval as in the case of biochemical examinations for diagnosing chronic diseases. Based on this consideration, our interpretation of the ELISA values indicated that 17 of the 1,869 individuals examined were positive, for an overall infection rate of 0.91%.
ing highly skewed data is illustrated by the histogram in Fig. 3, where the normal, Gaussian distribution of the serologic data permits valid statistical analysis.

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REFERENCES