[Jpn. J. Parasitol., Vol. 42, No. 6, 484–492, December, 1993]

Seroepidemiologic Studies on Amebiasis in Grand Recife, a Possible Endemic Area of Nonpathogenic *Entamoeba histolytica*, Pernambuco, Brazil

SEIKI KOBAYASHI[1,2], RICARD A. FONSECA[2], CYNTHIA M. ANDRADE[2], VILMA M. DE MELO[2], HIROSHI TACHIBANA[1,4], NOBUAKI KANEKO[1,2], SEIKI TATENO[1,3] AND TSUTOMU TAKEUCHI[1]

(Accepted for publication; December 8, 1993)

Abstract

Seroepidemiologic studies on *Entamoeba histolytica* infection were carried out in the region of Grand Recife, northeast Brazil. Serum and stool specimens were collected from institutionalized children in Recife and Paulista City, school children at Tururu and Inocoop in Paulista and Japanese immigrants in Recife. Stool examination revealed that 87 out of the 634 individuals tested were positive for *E. histolytica* cyst. The gel diffusion precipitin test (GDP) conducted for 558 serum specimens from these individuals was judged positive for only 1 Japanese immigrant with mild gastrointestinal symptoms as well as ameba cyst in the stool, who had moved from an endemic area of invasive amebiasis. However, the enzyme-linked immunosorbent assay (ELISA) was positive for 72 individuals (12.9%). For 42 asymptomatic cyst carriers selected from these subjects, the indirect immunofluorescent antibody test, Western blotting and indirect hemagglutination test with the antigen from pathogenic ameba (strain HM-1: IMSS) as well as a commercial dot ELISA were also applied in addition to GDP and ELISA. Among these procedures, Western blotting revealed the highest positive rate (76.2%), while virtually none of the other procedures were judged positive except for ELISA. The Japanese immigrant with positive GDP response showed positive reactions to all of the remaining serologic methods. A strain of *E. histolytica* isolated from an institutionalized child could produce amebic liver abscess in a hamster, although this individual was negative by all of these serologic procedures except for Western blotting. The ameba was isolated from this experimental abscess lesion and its antigenic property was found to conform to that of *E. histolytica* as examined by immunoperoxidase reaction. These observations suggest that this region was considered to be an endemic area of nonpathogenic amebae as far as judged from the data by the serologic methods. Our present study also suggests that even asymptomatic cyst carriers with negative GDP are often judged positive by the Western blotting and ELISA, though specificity and significance of these positive reactions need to be clarified. Moreover, the finding that *E. histolytica* isolated from a serologically negative individual could produce an experimental liver abscess in hamster seemed interesting to know correlation of pathogenicity and virulence of this protozoon.

Key words: *Entamoeba histolytica*, Asymptomatic cyst carrier, Serology, Pathogenicity, Brazil

[1]*Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, Shinjuku-ku, Tokyo 160, Japan.*
[2]*Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Recife, PE, Brazil.*
[3]*Division of International Medical Cooperation, International Medical Center of Japan, Shinjuku-ku, Tokyo 160, Japan.*
[4]*Department of Infectious Diseases, School of Medicine, Tokai University, Isehara, Kanagawa Pref. 259-11, Japan.*
小林正規　金子信明（慶応義塾大学医学部熱帯医学・寄生虫学教室，ペルナムグコ大学免疫病理センター（LIKA））
Ricard A. Fonseca Cynthia M. Andrade Vilma M. de Melo （ペルナムグコ大学免疫病理センター（LIKA））
橘　裕司（慶応義塾大学医学部熱帯医学・寄生虫学教室，東海大学医学部感染症学教室）
建野正毅（慶応義塾大学医学部熱帯医学・寄生虫学教室，国立国際医療センター国際医療協力部）
竹内　勤（慶応義塾大学医学部熱帯医学・寄生虫学教室）

Introduction

According to the previous reports by Sargeaunt (1987; 1988) on zymodemes of more than 6,000 isolates of *Entamoeba histolytica*, most of the asymptomatic cyst carriers were attributed to infection with the nonpathogenic strains. Their zymodeme studies also clarified the presence of such countries as Finland and Kuwait, where only nonpathogenic *E. histolytica* has been detected (Sargeaunt *et al.*, 1984). In Recife, northeast Brazil, *E. histolytica* cyst has been frequently detected by general stool examination, and the positive implication of this protozoan infection in diarrheal diseases has been strongly suggested; however, seroepidemiologic study by Okazaki *et al.* (1988) demonstrated that none of the asymptomatic individuals examined in this city, who were found to excrete *E. histolytica* cyst in stool, were judged positive by the gel diffusion precipitin test (GDP). In addition, they showed that only 9 out of 66 asymptomatic cyst carriers were positive by a more sensitive serologic method, enzyme-linked immunosorbent assay (ELISA). More recently, Nozaki *et al.* (1990) found only nonpathogenic zymodemes when they examined isoenzyme profiles of the ameba isolates in this city. All of these findings suggest that Recife is an endemic area of nonpathogenic *E. histolytica*. However, since diarrheal diseases are certainly serious health hazards particularly against children in the area around Recife, we felt it necessary to continue seroepidemiologic analysis on amebiasis, as it is possible that pathogenic amebae can be transferred from other regions like Amazon basin, where pathogenic *E. histolytica* has been previously detected (Araujo *et al.*, 1988; Batista *et al.*, 1988; Nozaki *et al.*, 1990). Moreover, it seemed also possible that a minor, small population of pathogenic *E. histolytica* may contaminate the larger number of nonpathogenic amebae in the culture for zymodeme analysis. The present investigation was attempted, therefore, to further confirm that this region is an endemic area of primarily nonpathogenic *E. histolytica* with children as the major target of investigation, since most of them had never been outside this region. If this is the case, the present seroepidemiologic study may be useful to know the reason for such an unique distribution profile of this protozoon in northeast Brazil.

Materials and Methods

Serum and stool specimens were collected from individuals in Recife and Paulista City in northeast Brazil as follows: (1) 170 children of 6–18 years of age from two institutions (Rodolfo Aureliano and Creche Jangadinha) in Recife; (2) 118 children of 2–9 years of age from an institution (Creche do Janga) in Paulista; (3) 251 school children of 6–12 years of age from two different communities (Tururu and Inocoop) in Paulista; (4) 95 Japanese immigrants of 3–73 years of age in Recife. Those belonging to group (1) to (3) had never been outside this region. Another serum specimen from a case with suspected amebic liver abscess in the Hospital das Clinicas, Universidade Federal de Pernambuco (UFPE), who had also never been outside Recife, was also examined in this study. In addition, 7 serum specimens from Brazilian cases with confirmed amebic liver abscess, kindly donated from the Instituto Medicina Tropical do Manaus, were employed as the positive control. As the negative control, sera from 6 students of School of Medicine, Keio University, Tokyo, who had no history of oversea travel and no gastrointestinal symptoms due to amebiasis as well as negative amebic serology by GDP and ELISA, were used. Moreover, serum specimens from Japanese immigrants in Recife, who were 6–12 years old and free from any clinical symptoms with negative amebic serology by GDP and ELISA as well as with negative stool examination for any intestinal parasites, were also employed as the negative control.

The gel diffusion precipitin test (GDP) was done according to the micro method reported by Maddison (1965) with minor modifications. The amebic antigen for GDP was prepared from axenically grown *E. histolytica* (strain HM-1: IMSS) of pathogenic zymodeme II in BI-S-33 medium (Diamond *et al.*, 1978) by preparing the crude extract of amebae at 3 mg protein/ml as described previously (Takeuchi and Kobayashi, 1983). The reaction in GDP was conducted utilizing 0.9% agarose L (Behring Institute, Germany) in veronal buffer (pH 8.2, μ=0.04) containing 0.1% sodium azide at the interwell distance of 3 mm. Evaluation was also done according to our previous method (Takeuchi and Kobayashi,

1983).

The enzyme-linked immunosorbent assay (ELISA) was essentially done as reported by Okazaki *et al.* (1988). The antigen was prepared from trophozoites (HM-1 strain) as described (Takeuchi and Kobayashi, 1983) to yield 10 μg soluble protein/ml in 50 mM carbonate buffer, pH 8.15. In this procedure, the antigen sensitization was carried out by adding 100 μl of the antigen solution per well utilizing a 96 well microtiter flat-bottled plate (Nunc Co., Denmark) followed by incubation fro 120 min at 35°C. Blocking was done for 2 hours utilizing 1% skim milk (Difco Laboratories, USA) containing 0.01% thimerosal in 150 mM phosphate-buffered saline, pH 7.2 (PBS) instead of 2% bovine serum albumin in the original procedure. Sample sera were diluted at 200-fold with the carbonate buffer, and horse raddish peroxidase (HRP)-labelled anti-human IgG rabbit immunoglobulin (Miles Laboratories, USA) was diluted at 10,000-fold by 50 mM PBS.

The dot ELISA was conducted utilizing a commercial kit (Amebiasis Dot-ELISA, LMD Laboratories, USA) following instructions by the manufacturer.

The indirect immunofluorescent antibody test (IFA) was done using HM-1 strain as the antigen grown as above. After the amebae were harvested, washed, and fixed in 3% formalin in 50 mM PBS at 4°C for 30 min, they were washed further thrice in the buffered saline. Subsequently, the amebae were placed, fixed to dryness onto a 10 well IFA slide glass (Foxy Brown Co., USA) at 500–1,000 amebae/well, and stored at –80°C until use. Blocking was done utilizing the skim milk containing thimerosal prepared as above in 10 mM PBS for 30 min at the room temperature. Sample sera were diluted at 4- to 4,096-fold by 10 mM PBS and added to the well, followed by incubation for 30 min at the same temperature. As the conjugated antibody, 50-fold diluted FITC-labelled anti-human IgG rabbit immunoglobulin (Miles Laboratories, USA) with 10 mM PBS was employed, and incubation was also done for 30 min at the same temperature. Serum specimens of distinct fluorescence at more than 64-fold dilution were judged positive.

The indirect hemagglutination test (IHA) was done utilizing a kit prepared and kindly donated by Dr. H. Yamaura in Tokyo Women's Medical College who fixed the soluble antigen prepared from HM-1 strain of amebae onto chicken red blood cells in the presence of 1% glutaraldehyde followed by freeze-thawing. The distinct agglutination of sensitized red blood cells at more than 80-fold serum dilution was judged positive according to Yamaura *et al.* (1985).

The Western immunoblotting was done utilizing Laemmli's buffer system (Laemmli, 1970) and 10% polyacrylamide gel. After the crude extract was prepared as above (Takeuchi and Kobayashi, 1983) at 50 μg protein/lane from trophozoites of HM-1 strain, solubilized by incubating in sodium dodecyl sulfate at 95°C for 5 min and transferred to a nitrocellulose membrane by the method of Towbin *et al.* (1979), the antibody was detected by the immunoperoxidase method mentioned above. Blocking was done by 3% skim milk containing 0.01% thimerosal in 10 mM PBS as above. As the conjugated antibody, the HRP-labelled anti-IgG immunoglobulin mentioned above (Miles Laboratories, USA), which was diluted 200-fold with 3% skim milk, was added, and the incubation was done for 60 min at the room temperature. The enzymatic reaction of peroxidase was visualized with 0.5% 4-chloro-1-naphtol as the substrate by incubating for 60 min at the same temperature. The positive reaction was determined by comparison with the data on negative controls.

The virulence of amebae isolated and maintained in Robinson's medium (Robinson, 1968) were evaluated utilizing an animal model. The amebae were isolated from 5 different asymptomatic cyst carriers with negative GDP response in Recife, grown in the medium and concentrated by centrifugation of the medium over Ficol (d=1.17) at 1,000 rpm × 10 min. Subsequently, the amebae were washed in a culture fluid (Boeck and Drbohlav, 1925) containing penicillin 10,000 units and streptomycin 10 mg/ml. Finally, these amebae were suspended in the medium with these antibiotics and injected into the left lobe of a syrian golden hamster of 40–60 g body weight at 20,000 amebae/head in amount of 0.1 ml. Abscess formation was confirmed 4 days later by the macroscopic observation under laparotomy. Subsequently, portions of the abscess lesion were resected and processed to yield conventional

hematoxylin-eosin stained histopathological slides after fixation in 10% formalin. Moreover, the antigenicity of amebae in the tissue sections was confirmed by reacting with anti-amebic antibody produced by immunization of a rabbit (2 kg) with the extract of trophozoites of HM-1 strain prepared as above (Takeuchi and Kobayashi, 1983) at 2 mg protein/head, twice in 2 weeks with 7 days interval, followed by the immunoperoxidase reaction to identify the antigenicity of amebae as reported by Kobayashi *et al.* (1985).

Stool examination was done by the conventional simple smear technique, and also by the concentration method using formalin-ether according to Ritchie (1948).

Results

Table 1 summarizes some demographic features of the individuals examined in this study and the number of subjects positive for *E. histolytica* cyst by stool examination. From these observations, it seems clear that except for the Japanese immigrants in Recife, all of the communities tested exhibited the high prevalence of *E. histolytica* infection. The highest prevalence was demonstrated at the Creche do Janga. However, virtually none of these stool examination-positive individuals showed any appreciable clinical symptoms, which could be attributable to this protozoan infection.

In Table 2, correlation of the results by stool examination with those of GDP and ELISA was summarized, which indicated that none of the individuals with *E. histolytica* cyst in stool were judged positive by GDP except for only one Japanese immigrant in Recife. Thus, it seems likely that among the Brazilian children, who had no experience of travel outside the region around Recife and Paulista, invasive amebiasis with a positive GDP response was quite infrequent. Concerning only one serologically positive Japanese immigrant, who was a 73 year old female, she had lived for 2 years at Manaus, an endemic area of invasive amebiasis, and moved to Recife about 30 years ago; accordingly, we could not exclude the possibility that she had been infected with this pathogen while she was in

Table 1 Prevalence of *Entamoeba histolytica* in six communities in Recife and Paulista City by stool examination

City	Community	No. examined	Age	No. cyst positive		
				Male (M)	Female (F)	Total
Recife	Rodolfo Aureliano	79 (M;52, F;27)	6~18	11 (21.2%)	4 (14.8%)	15 (19.0%)
	Creche Jangadinha	91 (M;61, F;30)	6~18	7 (11.5%)	4 (13.3%)	11 (12.1%)
	Japanese Immigrants	95 (M;52, F;43)	3~73	0	1 (2.3%)	1 (1.1%)
Paulista	Creche do Janga	118 (M;70, F;48)	2~9	16 (22.9%)	15 (31.3%)	31 (26.3%)
	Tururu	143 (M;76, F;67)	6~12	6 (7.9%)	14 (20.9%)	20 (14.6%)
	Inocoop	108 (M;59, F;49)	6~12	2 (3.4%)	7 (14.3%)	9 (8.3%)
Total		634 (M;370, F;264)		42 (11.4%)	45 (17.1%)	87 (13.7%)

Table 2 Serologic examination for amebic infection by the gel diffusion precipitin test (GDP) and enzyme-linked immunosorbent assay (ELISA) in Recife and Paulista, northeast Brazil

Community	Cyst in stool	GDP		ELISA		No. examined
		+	–	+	–	
Rodolfo	+	0	15	4	11	15
Aureliano	–	0	46	7	39	46
Creche	+	0	14	4	10	14
Jangadinha	–	0	69	14	55	69
Creche do	+	0	22	6	16	22
Janga	–	0	53	6	47	53
Tururu	+	0	18	6	12	18
	–	0	120	13	107	120
Inocoop	+	0	9	0	9	9
	–	0	97	8	89	97
Japanese	+	1	0	1	0	1
immigrants	–	0	94	3	91	94
		1	557	72	486	558

Manaus. The positive rate by ELISA ranged 4.2 to 21.7%; the highest rate at the Creche Jangadinha, while the lowest at the Japanese immigrants.

Table 3 summarizes the comparative data by six different serologic tests on the 42 asymptomatic cyst carriers selected from Rodolfo Aureliano, Creche Jangadinha and Creche do Janga. Data on the 7 positive controls obtained at Manaus were also demonstrated for comparison. These studies indicated that GDP, IFA and dot-ELISA were judged

Table 3 Serologic examination of asymptomatic cyst carriers of *Entamoeba histolytica* by six different procedures

Methods	No. positive/No. examined			
	Asymptomatic cyst carriers	Positive Control	Case 1	Case 2
GDP	0/42	7/7	1/1	1/1
ELISA	12/42	7/7	1/1	1/1
Dot-ELISA	0/42	2/2	1/1	1/1
IFA	0/39	2/2	1/1	1/1
IHA	1/42	7/7	1/1	0/1
Western blotting	32/42	7/7	1/1	1/1

Positive control: Sera from the cases of Inst. Med. Trop. Manaus
Case 1: Amebic colitis suspected from Japanese immigrants
Case 2: Amebic liver abscess suspected at the Hopital das Clinicas, UFPE
Abbreviations of the methods were given in the text.

negative for all of these individuals, while the positive rate by Western blotting reached 76% and was much higher than that of each of the other 5 methods. On the other hand, anti-amebic antibody was detectable in the patients with invasive amebiasis employed as the positive control by all these 6 serologic procedures. In regard to the case from the Hospital das Clinicas of UFPE, IHA was judged negative, whereas all of the other methods showed positive reaction, which suggested that this patient can be diagnosed as amebic liver abscess. The negative controls were judged negative by all of the procedures employed including Western blotting in addition to GDP and ELISA.

To further examine if such a high positive rate by Western blotting was due to nonspecific reaction by some concomitant infection, we selected 13 sera from the individuals from these communities, who were asymptomatic and positive for *Giardia lamblia* cyst but not *E. histolytica*. We also isolated 12 sera from the individuals from whom the culture of *E. histolytica* could be established utilizing Robinson's medium. Lack of the infection with other intestinal parasites from these 12 individuals was also confirmed. Western blotting on the former 13 sera showed that 5 of them were positive, while 11 out of the 12 sera from the latter were judged positive by the same procedure, which made a statistically significant difference (data not shown).

Correlation of Western blotting and ELISA on some of the asymptomatic cyst carriers listed in Table 3 as well as the positive and negative controls was demonstrated in Fig. 1. It seemed evident that all of the ELISA-positives were also judged positive by Western blotting. However, there were some cases who were judged positive by the blotting but not by ELISA.

In this study, virulence of the isolated strains of *E. histolytica* from serologically negative asymptomatic cyst carriers was also evaluated. As demonstrated in Fig. 2, an experimental amebic liver abscess in hamster (5–7 mm diameter) was induced by inoculating the amebae isolated from a GDP-negative individual at the Creche do Janga. Identification of the organisms by the immunoperoxidase method on the tissue specimen from this experimentally induced liver abscess showed that they were distinctly stained with the enzymatic reaction in the

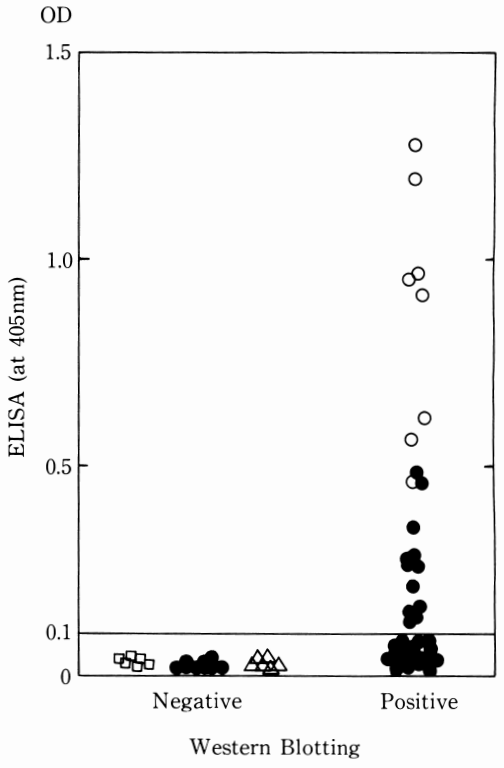

Fig. 1 Correlation of the Western blotting and enzyme-linked immunosorbent assay (ELISA) on asymptomatic cyst carriers of *Entamoeba histolytica* with negative response to the gel diffusion precipitin test
□ : Negative control (students of Keio University)
△ : Negative control (children selected from Japanese immigrants in Recife)
○ : Positive control (cases with amebic liver abscess from Manaus and a case with suspected amebic colitis from Japanese immigrants in Recife)
● : Asymptomatic cyst carriers tested from children in Recife and Paulista, northeast Brazil
The horizontal line at around OD 0.1 (0.109) in the ELISA stands for the cut off value (the upper limit of 99% critical range) calculated from the data on negative controls.

presence of anti-amebic antibody (Fig. 3), which suggests that *E. histolytica* was certainly responsible for the abscess formation in this experimental model.

Discussion

Serologic tests have been useful for the effective

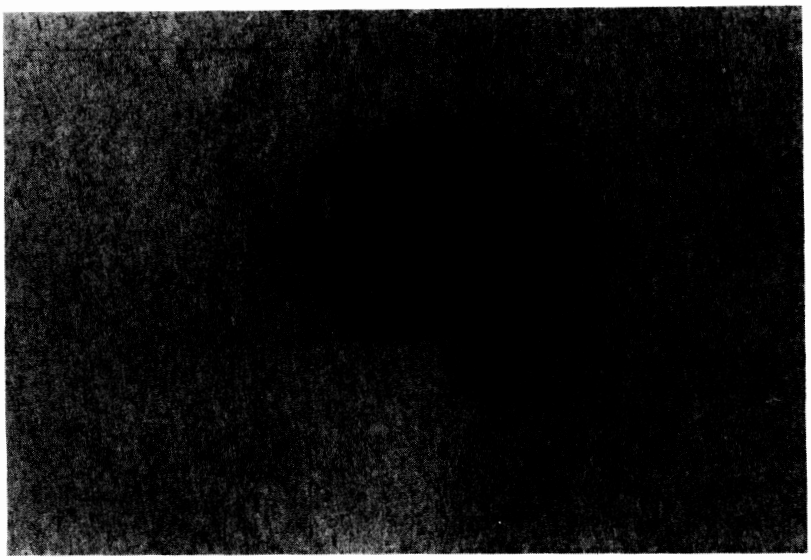

Fig. 2 A macroscopic view of the liver of hamster experimentally inoculated with *E. histolytica* isolated from an asymptomatic cyst carrier with negative amebic serology. Arrow head shows the abscess lesion. Other details as in the text.

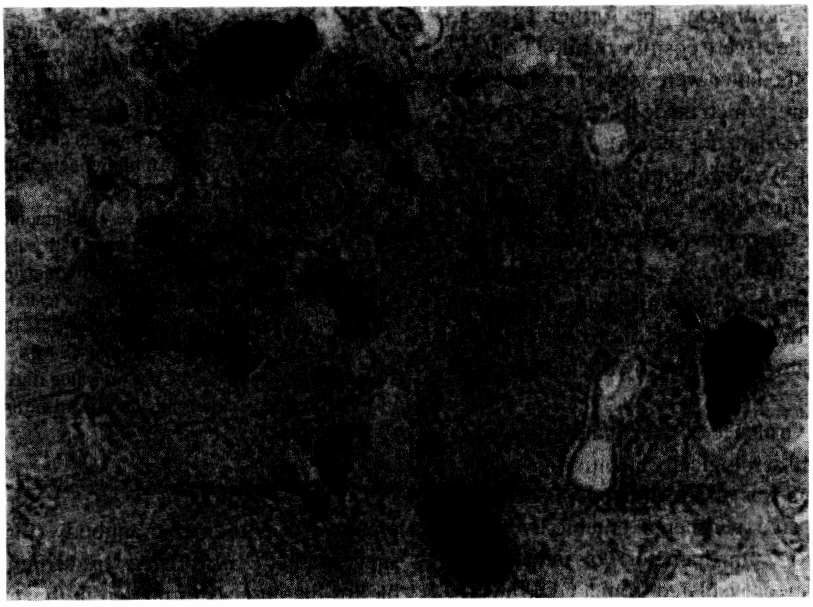

Fig. 3 A light microscopic view of the abscess lesion as examined by the immunoperoxidase method. The positive reaction can be visualized by deposit of the reaction product of peroxidase (arrows.) (×400)

diagnosis of invasive amebiasis, while they have been considered little reliable against noninvasive amebic infection, mostly asymptomatic cyst carriers. This may be overcome by using highly sensitive serologic procedures like Western blotting and ELISA, although Sargeaunt (1987) defined asymptomatic cyst carriers with *E. histolytica* of the nonpathogenic zymodemes as those lacking production of detectable anti-amebic antibodies.

Indeed, there is a possibility that sensitive serologic methods may detect nonspecific antibodies, in particular when concomitant infections with other intestinal protozoa are present, as Tachibana *et al.* (1990) showed that they could produce monoclonal antibodies which recognized the antigenic epitopes commonly present not only in *E. histolytica* but also in *G. lamblia, Trichomonas vaginalis* and Hela cells. In the present study, serologic examination for *E. histolytica* infection in Recife and Paulista indicated that Western blotting revealed an extremely high positive rate for the asymptomatic cyst carriers with negative GDP, IFA and dot-ELISA responses, which led us to conceive that they were infected with nonpathogenic *E. histolytica* on the basis of the view by Jackson (1987). Examination carried out to know if such a high positive rate by Western blotting was ascribed to the concomitant infections suggested that *G. lamblia* infection could not entirely account for the high serologically positive response of asymptomatic cyst carriers of *E. histolytica* by this method. These findings suggest that Western blotting may be worth further evaluation against asymptomatic cyst carriers of *E. histolytica*; thus, it seems possible that some sorts of low-level of anti-amebic antibody might be produced even when only nonpathogenic amebae infect humans, although its specificity and pathophysiological significance should be evaluated carefully.

Previously Nozaki *et al.* (1990) carried out zymodeme analysis on the isolates of *E. histolytica* in the same northeast region of Brazil, and detected only nonpathogenic zymodemes in the area of Grand Recife, while they detected pathogenic zymodemes in the area of Grand Recife, while they detected pathogenic zymodemes in Manaus and Belem. On the other hand, Cunha *et al.* (1977a; b; c) conducted epidemiological study on amebic infection in 3 different areas of Brazil and detected symptomatic amebiasis at Grand Belo Horizonte and Galiléia in the State of Minas Gerais, and at Macapá in the State of Amapá by serologic procedures and endoscopy. Cuadrado and Kagan (1967) also carried out a seroepidemiologic analysis on amebic infection in 7 different locations in Brazil, and reported that the serologically positive rate of army soldiers at the northeast region of Brazil was lower than those in the Amazon Basin and the State of Minas Gerais. These previous data appear to be well in accord with our present observations as judged from the previous view by Jackson (1987) suggesting that GDP was highly correlated with zymodemes of *E. histolytica*. However, according to the development of traffic measures in Brazil, invasion of pathogenic amebae into the northeast region might be inevitable in near future. Rather, our present symptomatic cases from Japanese immigrants and the Hospital das Clinicas of UFPE may indicate that invasion of pathogenic amebae had already occurred. Sargeaunt (1985) and Blanc *et al.* (1989) previously suggested that pathogenic and nonpathogenic *E. histolytica* did not coexist in a single clinical specimen, which may explain why nonpathogenic amebae are predominant in the northeast region of Brazil. However, it seems also possible that a small, minor population of pathogenic amebae can be concomitantly present with larger population of nonpathogenic *E. histolytica* in a single host, which can not be assessed by the conventional isoenzyme analysis. To further evaluate such a possibility, we are now trying to apply monoclonal antibodies for detecting a small number of pathogenic amebae in the larger population of nonpathogenic *E. histolytica* in the culture.

According to Burchard and Mirelman (1988), there were both virulent and avirulent strains in pathogenic and nonpathogenic *E. histolytica*. In the present study, we have observed that one of the 5 strains isolated from the asymptomatic cyst carriers could produce an experimental amebic liver abscess in a hamster. This may not directly correlate with the virulence against humans; however, this observation should be paid attention to know the virulence of amebae in this region, as the individual from whom this ameba was isolated was serologically negative by GDP, IFA and dot-ELISA. It seems also

possible that in contrast to the previous view by Sargeaunt (1985) and Blanc *et al.* (1989) pathogenic and nonpathogenic *E. histolytica* can coexist in a single host.

References

1) Araujo, R., Cruz, E. M. and Araujo, M. T. F. (1988): Estudo anatomo patologico da amebíase em Belem do Para. Suppl. XXIV Congr. Soc. Brasil. Med. Trop., 141.
2) Batista, E., Neto, L., Guerra, A. and Gomes, G. (1988): Estudo clínico de abscesso hepático em crianças. Suppl. XXIV Congr. Soc. Brasil. Med. Trop., 141.
3) Blanc, D., Nicholls, R. and Sargeaunt, P. G. (1989): Experimental production of new zymodemes of *Entamoeba histolytica* supports the hypothesis of genetic exchange. Trans. R. Soc. Trop. Med. Hyg., 83, 787–790.
4) Boeck, W. C. and Drbohlav, J. (1925): The cultivation of *Entamoeba histolytica*. Am. J. Hyg., 5, 371–407.
5) Burchard, G. D. and Mirelman, D. (1988): *Entamoeba histolytica*: Virulence potential and sensitivity to metronidazole and emetine of four isolates possessing nonpathogenic zymodemes. Exp. Parasitol., 66, 231–242.
6) Cuadrado, R. R. and Kagan, I. (1967): The prevalence of antibodies to parasitic diseases in sera of young army recruits from the United States and Brazil. Am. J. Epidem., 86, 330–340.
7) Cunha, A. S., Raso, P., Silva, E. F. and Mello, S. M. (1977a): Patogenia da amebíase. II-Endoscopia e histopatologia da mucosa retossigmoidiana na amebíase intestinal. Rev. Inst. Med. Trop. São Paulo, 19, 378–386.
8) Cunha, A. S., Silva, E. F., Araujo, F. G., Costa, C. A., Mello, S. M. and Raso, P. (1977b): Patogenia da amebíase. III-Comportament de provas serológicas em pacientes infectados pela *Entamoeba histolytica*. Rev. Inst. Med. Trop. São Paulo, 19, 387–392.
9) Cunha, A. S., Silva, E. F., Raso, P. and Mello, S. M. (1977c): Patogenia da amebíase. I-Aspectos da amebíase no Brasil. Estudo realizado em três grupos populacionais de três regiões geograficas distintas. Rev. Inst. Med. Trop. São Paulo, 19, 289–300.
10) Diamond, L. S., Harlow, D. R. and Cunnick, C. C. (1978): A new medium for the axenic cultivation of *Entamoeba histolytica* and other Entamoebae. Trans. R. Soc. Trop. Med. Hyg., 72, 431–432.
11) Jackson, T. F. H. G. (1987): *Entamoeba histolytica* cyst passers-to treat or not to treat? S. Afr. Med. J., 72, 657–658.
12) Kobayashi, T., Koretoh, O., Kamachi, M., Watanabe, S., Ishigoka, S., Matsushita, I. and Sawaragi, I. (1985): Cytologic demonstration of *Entamoeba histolytica* using immunoperoxidase techniques. Report of two cases. Acta Cytol., 29, 414–418.
13) Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680685.
14) Maddison, S. E. (1965): Characterization of *Entamoeba histolytica* antigen-antibody reaction by gel diffusion. Exp. Parasitol., 16, 224–235.
15) Nozaki, T., Aca, I., Okuzawa, E., Magalhães, M., Tateno, S. and Takeuchi, T. (1990): Zymodemes of *Entamoeba histolytica* isolated in the Amazon and the north-east of Brazil. Trans. R. Soc. Trop. Med. Hyg., 84, 387–388.
16) Okazaki, M., Okazaki, M., Miranda, P., Neto, J., Doegues, V., Alves, J., Cauas, M., Tanabe, M., Kobayashi, S., Kaneko, N., Nagakura, K., Kobayashi, M., Motta, S. R. N., Tateno, S. and Takeuchi, T. (1988): Parasitological and serological studies on amoebiasis and other intestinal parasitic infections in Recife and its suburban area, northeast Brazil. Rev. Inst. Med. Trop. São Paulo, 30, 313–321.
17) Pessoa, S. B. and Martins, A. V. (1978): Pessoa parasitologia medica. 10th ed., Editoria Guanabara Koogans, A.-RJ., 918–919.
18) Ritchie, L. S. (1948): An ether sedimentation technique for routine stool examination. Bull. US. Army Med. Dept., 8, 326–328.
19) Robinson, G. L. (1968): The laboratory diagnosis of human parasitic amoebae. Trans. R. Soc. Trop. Med. Hyg., 62, 285–294.
20) Sargeaunt, P. G. (1987): The reliability of *Entamoeba histolytica* zymodemes in clinical diagnosis. Parasitol. Today, 3, 40–43.
21) Sargeaunt, P. G. (1988): Zymodemes of *Entamoeba histolytica*. in Amebiasis, Ravdin, J. I. ed., John Wiley and Sons, New York, 370–387.
22) Sargeaunt, P. G., Baveja, U. K., Nanda, R. and Anand, B. S. (1984): Influence of geographical factors in the distribution of pathogenic zymodemes of *Entamoeba histolytica*: identification of zymodeme XIV in India. Trans. R. Soc. Trop. Med. Hyg., 78, 96–101.
23) Tachibana, H., Kobayashi, S., Nagakura, K., Kaneda, Y. and Takeuchi, T. (1990): Identification of pathogenic isolate-specific 30,000-Mr antigen of *Entamoeba histolytica* by using a monoclonal antibody. Infect. Immun., 58, 955–960.
24) Takeuchi, T. and Kobayashi, S. (1983): Serologic diagnosis of amoebiasis. Immuno-Adv., 12, 27–30 (in Japanese).
25) Towbin, H., Stahelin, T. and Gordon, J. (1979): Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci., 76, 4350–4354.
26) Yamaura, H., Shirasaka, R., Sato, J., Odagiri, Y., Iseki, M., Kimata, I., Maeno, Y., Nakamura, O. and Totani, T. (1990): Application of the indirect hemagglutination test using glutaraldehyde-fixed chicken red blood cells to serological diagnosis of amebiasis. Jpn. J. Parasitol., 39, 468–474.