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Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in cyst-passers by immunoblot

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Abstract: Differentiation of invasive strains of *Entamoeba histolytica* according to their pathogenicity has been a topic of long debate, but now the pathogenic species only is regarded as *E. histolytica* while the non-pathogenic species is *E. dispar*. The present study applied immunoblot to differentiate infections of the two species among microscopically-detected cyst-passers in Korea. The crude extract of *E. histolytica* separated in 5-20% gradient gels, revealed many fractions of 94, 81, 71, 50, 44, 38.5, 37.5, 29, 19, and 18 kDa when the cysteine proteinase inhibitor, E64, was supplemented. The serum IgG antibody of 3 proven *E. histolytica* cases reacted with the antigenic fractions of 117, 110, 99, 68, 66, 60, 54, 52, 46, and 45 kDa. Sera of PCR confirmed 3 cases of *E. dispar* reacted only to the 117 kDa fraction of the *E. histolytica* crude extract which was regarded as non-specific. To the antigen of monoxenic *E. dispar*, sera of *E. dispar* and *E. histolytica* cases showed the same immunoblot reactions. The serum IgA antibody reacted with several antigenic fractions of both *E. histolytica* and *E. dispar*, but IgM and IgE antibodies showed no reaction to either antigen. Sera of 24 symptomless amebic cyst-passers were screened with the *E. histolytica* antigen; two were found to be infected by *E. histolytica* and 22 were by *E. dispar*. The present findings suggest that in Korea most of asymptomatic cyst passers of *E. histolytica* are carriers of *E. dispar*. Immunoblot using *E. histolytica* antigen is a good technique for the differentiation of *E. histolytica* and *E. dispar* infections.
Key words: *Entamoeba histolytica*, *Entamoeba dispar*, immunoblot, IgG antibody

INTRODUCTION

Entamoeba histolytica is a pathogen which is worldwidely prevalent in people who live in poor hygiene. About ten percent of infected cases are known to manifest clinical symptoms. This discrepancy between high prevalence and low morbidity is explained by the two species; pathogenic *E. histolytica* and non-pathogenic *E. dispar*. The two species are morphologically identical, but are differentiated by immunologyic, biochemical, pathogenic, and genetic characteristics (Diamond and Clark, 1993). Since infection by *E. dispar* is known to be non-harmful, it is practically necessary to distinguish it from that by *E. histolytica* (Tannich and Buchard, 1991).

The diagnosis of amebic colitis or liver abscess is rather tricky. Fresh specimens should be examined to detect trophozoites in the acute phase of colitis and the recovery of trophozoites in liver aspirates is almost impossible. Even if the trophozoites or cysts are recovered in clinical materials, species identification of *E. histolytica* by isoenzyme

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analysis, PCR and RFLP, and staining with monoclonal antibodies is necessary. Several groups of researchers have produced monoclonal antibodies against such as 125 kDa, 30 kDa, 29 kDa peptides or FI antigen to differentiate the two species. The MAbs against a highly immunogenic fraction of *E. histolytica* made use of the detection of *E. histolytica* antigen in stool, though it is possible that only 8 ng of this antigen was present (Sengupta *et al.*, 1993; Montfort *et al.*, 1994). However, this method is applicable only to intestinal amebiasis. As a differentiation method of *E. histolytica* from *E. dispar*, the use of PCR and RFLP of P1 gene is also promising (Tannich and Buchard, 1991). However, PCR is possible only if ameba DNA is readily extracted.

In such context, serology has proved to be a useful aid to clinical diagnosis, and especially in patients with hepatic amebiasis, ELISA has been applied, but the use of ProSpecT\ⓣ/ microplate enzyme immunoassay cannot differentiate between *E. histolytica* and *E. dispar* (Ong *et al.*, 1996). Shenai *et al.* (1996) reported good sensitivity and specificity by ELISA using recombinant antigen of the 170 kDa subunit lectin.

This study was undertaken to evaluate immunoblot in differentiating *E. histolytica* from *E. dispar*.

MATERIALS AND METHODS

Antigens

E. histolytica the YS-27 strain (Chang *et al.*, 1995) from an amebic liver abscess patient was cultured axenically in TYI-S-33 medium with 15% adult bovine serum. *E. dispar* S16 (Choe *et al.*, 1996) isolated at the Seoul Paik Hospital was grown in TYS GM-9 monophasic medium containing *Escherichia coli* LE392. These isolates were rinsed three times in cold phosphate-buffered saline (PBS), and were supplemented with proteinase inhibitors: L-trans-epoxy-succinyl-leucylamide-(4-guanidino)-butane, N-[N-(L-3-transcarboxy-irane-2-carbonyl)-L-leucyl]-agmatine (E64, 10 μM, Sigma, USA), iodoacetate (iodoacetic acid, 100 M, Sigma, USA), pepstatin A (pepstatin, 1 μM, Sigma, USA) and phenylmethane-sulphonyl fluoride (PMSF, 1 mM, Sigma, USA). They were then centrifuged at 12,000 rpm for 60 min, and the supernatant was stored at -70°C until required for use as antigen.

Sera

Sera were obtained from three individuals infected wity *E. histolytica*, and from three with *E. dispar* infection confirmed by PCR and RFLP (Choe *et al.*, 1996). Sera used as negative control were obtained from ten healthy cyst-negative individuals. The sera of *Entamoeba coli* and *Blastocystis hominis* infections were collected from positive cases detected by routine stool examination in Seoul National University Hospital. Sera from 24 microscopically confirmed cases of *E. histolytica*, who were positive for the cyst in their stool, were screened by immunoblot to differentiate between *E. histolytica* and *E. dispar* infections.

SDS-PAGE

To observe the effect of several proteinase inhibitors, soluble extracts supplemented with several proteinase inhibitors were separated in 5-20% gradient resolving gels (Laemmli, 1970). The stacking gel was 3% and a constant current of 20 mA was supplied. The gel was stained with Coomassie blue R-250.

Enzyme-linked immunoelectrotransfer blot (immunoblot)

Forty μg of soluble protein on 10% polyacrylamide-sodium dodecyl sulfate slab gels was separated in a well by electrophoresis and then transferred to a nitrocellulose membrane (Tsang *et al.*, 1983). After the transfer, the membrane was cut into strips and blocked for nonspecific binding with 3% skim milk (Difco Laboratories, Detroit, Mich.) for 2 hours and then washed in phosphate-buffered saline with 0.1% Tween 20 for 30 minutes. The strips were allowed to react with primary antibody (diluted 1:100) for 2 hours, and then with secondary antibody, peroxidase-conjugated goat affinity purified antibody to human IgG, IgM, IgA, and IgE antibodies (Cappel, U.S.A., diluted 1:1000) respectively for 1 hour. After extensive washing, the substrate, 0.6% chloronaphthol, 0.2% diaminobenzidine and 0.02% H_2O_2/PBS, was

applied for color development.

RESULTS

Protein fractions of *E. histolytica* and *E. dispar* crude extracts on SDS-PAGE

Crude extracts of YS-27 *E. histolytica* trophozoites were supplemented with several proteinase inhibitors, and separated by 5-20% gradient SDS-PAGE (Fig. 1). The Coomassie blue stained protein fractions supplemented by a serine proteinase inhibitor (PMSF) were 67, 50, 44, 38.5, 37.5, 29, and thick bands around 10 kDa. The extract supplemented by a cysteine proteinase inhibitor (E64), resolved more than 20 bands in 10 to 266 kDa range. The major fractions were 94, 81, 71, 50, 44, 38.5, 37.5, 29, 19 and 18 kDa. The crude extracts, either treated with an aspartic proteinase inhibitor (pepstatin A) or no proteinase inhibitor, showed the same patterns of peptide bands at 71, 44, 38.5, 37.5, and 29 kDa. This finding strongly suggests that the activity of cysteine proteinase of *E. histolytica* is higher than that of other proteinases.

Crude extract of monoxenic cultured *E. dispar* separated by 5-20% gradient SDS-PAGE were stained by Coomassie blue R-250 (Fig. 2). More than 30 peptide fractions were detected from crude extract of *E. dispar*, the major ones were at 140, 118, 69, 66, 56, 52, 49.2, 44, 40 and 14 kDa.

Antigenic fractions of *E. histolytica*

1) Anti-ameba IgG antibody: In crude antigen of *E. histolytica* supplemented with E64, human sera of confirmed *E. histolytica*

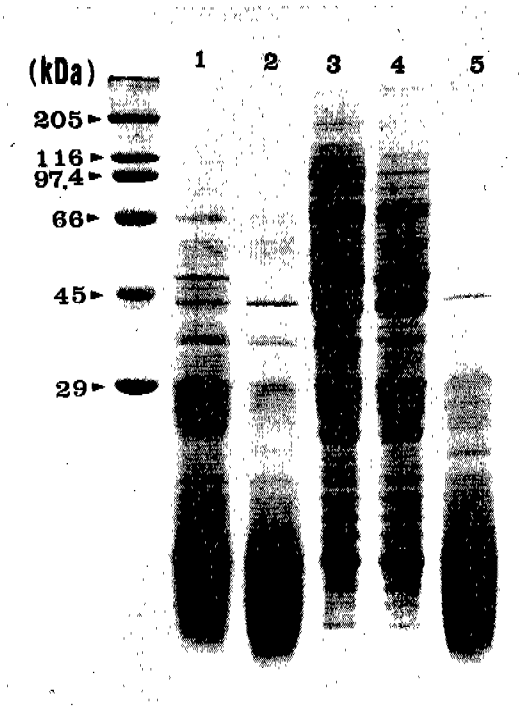

Fig. 1. The effects of proteinase inhibitors on crude extracts of *E. histolytica* YS-27 trophozoites by SDS-PAGE in a 5-20% gradient gel. Lane 1, serine proteinase inhibitor (PMSF); lane 2, aspartic proteinase inhibitor (pepstatin A); lane 3, cysteine proteinase inhibitor (E64); lane 4, cysteine proteinase inhibitor (iodoacetic acid); lane 5, without proteinase inhibitors.

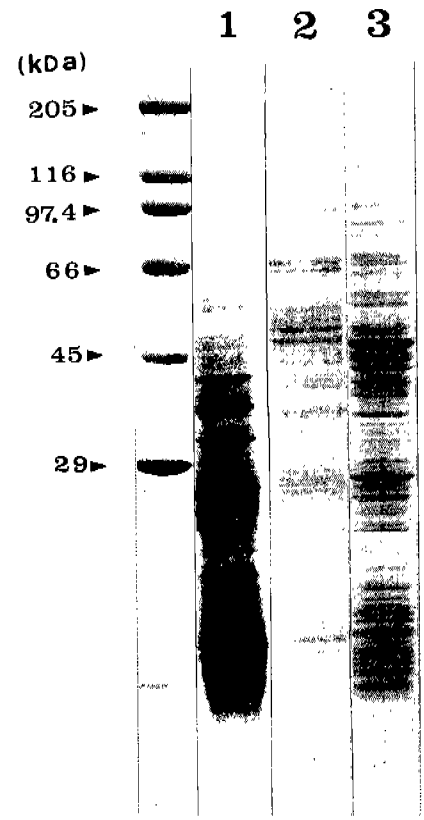

Fig. 2. Comparison of crude extracts of *E. histolytica* and *E. dispar* separated in 5-20% gradient SDS-PAGE. Lane 1, *E. histolytica* (YS-27); lane 2, *E. dispar* (S16); lane 3, *Escherichia coli*.

infection reacted to the 110, 99, 68, 66, 60, 54, 52, 46 and 45 kDa fractions. Contrary to this, *E. dispar* infection reacted only to the 117 kDa fraction (Fig. 3). The anti-sera from infection cases of *E. coli*, *B. hominis* and normal control also reacted to the 117 kDa band of *E. histolytica*. In iodoacetic acid supplemented crude antigen of *E. histolytica*, anti-*E. dispar* sera showed no response compared with several bands without any common band to anti-*E. histolytica* sera. The human sera of *E. histolytica* infection showed no response to crude antigen of *E. histolytica* which was supplemented with serine, aspartic acid proteinase inhibitor (PMSF and pepstatin A), or non-proteinase inhibitor.

2) Anti-ameba IgA antibody: In E64 supplemented crude antigen of *E. histolytica*, sera of *E. histolytica* patients reacted to the 180, 174, 160, 110, 103, 99, 68, 66, 60, 54, 52, 46 and 45 kDa fractions, while 110, 103, 99, 68, 66 and 60 kDa fractions were strong. Serum reactions of *E. dispar*, *E. coli*, or *B. hominis* infection showed the same pattern as that of *E. histolytica* infection (Fig. 4).

In iodoacetic acid supplemented crude antigen of *E. histolytica*, sera of *E. histolytica* patients reacted to the 110, 103, 99, 68, 66, 60, 54, 52, 46 and 45 kDa fractions. In PMSF-supplemented crude antigen of *E. histolytica*, sera of *E. histolytica* patients reacted to the 117, 86, 79, 66, 54, 50, 49, 43, 37 and 31 kDa fractions, and sera of *E. dispar* infections reacted to the 49 kDa band. The sera of *E. coli* or *B. hominis* infection reacted to the 66 and 49 kDa fractions. In crude antigen of *E. histolytica* supplemented with pepstatin A or no proteinase inhibitors, there was no reaction.

3) Anti-ameba IgM antibody: Any serum showed no reaction to crude antigens of *E. histolytica*.

4) Anti-ameba IgE antibody: Any serum showed no reaction to crude antigens of *E. histolytica*.

Antigenic fractions of *E. dispar*

1) Anti-ameba IgG antibody: In E64 supplemented crude antigen of *E. dispar*, sera of *E. dispar* infection reacted to the 138, 128, 90, 73, 63, 56, 55, 52, 50 and 43 kDa bands,

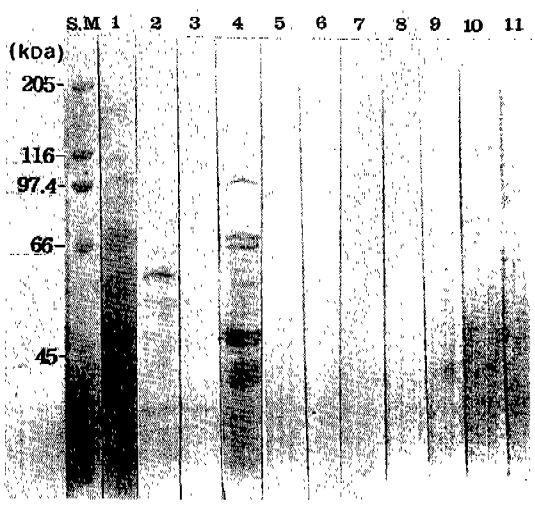

Fig. 3. Immunoblot patterns of serum IgG antibodies to the E64 supplemented crude extracts of *E. histolytica*. Lane 1, amido black 10B-stained protein fractions; lanes 2-4, sera of *E. histolytica* patients; lanes 5-8, sera of *E. dispar* infected humans; lane 9, serum of *E. coli* infection; lane 10, serum of *B. hominis* infection; lane 11, serum of normal control.

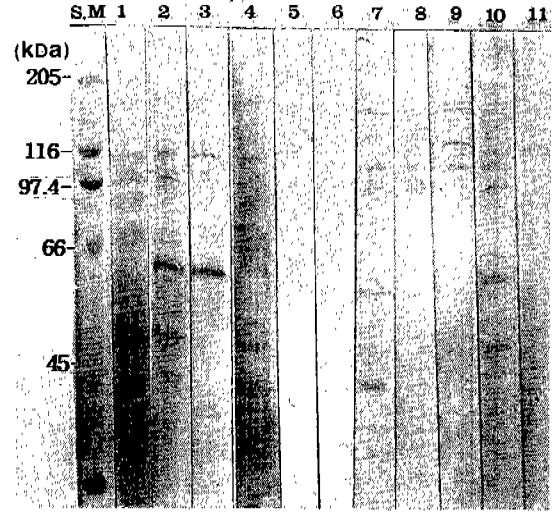

Fig. 4. Immunoblot patterns of serum IgA antibodies to the E64 supplemented crude extracts of *E. histolytica*. Lane 1, amido black 10B-stained protein fractions; lanes 2-4, sera of *E. histolytica* patients; lanes 5-8, sera of *E. dispar* infected humans; lane 9, serum of *E. coli* infection; lane 10, serum of *B. hominis* infection; lane 11, serum of a normal control.

and anti-*E. histolytica* sera reacted to the 138, 128, 115, 96, 90, 73, 63, 56, 55, 52, 50 and 43 kDa bands (Fig. 5).

2) Anti-ameba IgA antibody: In E64 supplemented crude antigen of *E. dispar*, anti-*E. dispar* and anti-*E. histolytica* sera reacted to the 132, 125, 98, 66, 62, 59, 56, 52, 50 and 43 kDa bands (Fig. 6).

Sera of humans microscopically positive for *E. histolytica* cysts in stool

In the sera of 24 cases whose stool was microscopically positive for *E. histolytica* cysts, two cases of *E. histolytica* and 22 of *E. dispar* were identified (Fig. 7). The sera of the *E. dispar* cases mostly showed reaction to the one band of 117 kDa, but those of *E. histolytica* cases reacted to most of the bands; 117, 110, 99, 68, 66, 60, 54, and 52 kDa.

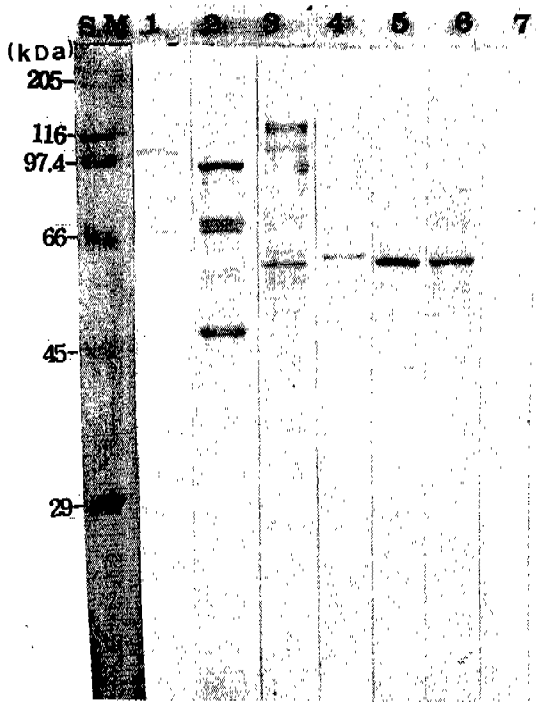

Fig. 5. Immunoblot patterns of serum IgG antibodies to the E64 supplemented crude extracts of *E. dispar*. Lanes 1-3, sera of *E. histolytica* infection; lanes 4-6, sera of *E. dispar* infection; lane 7, serum of a control.

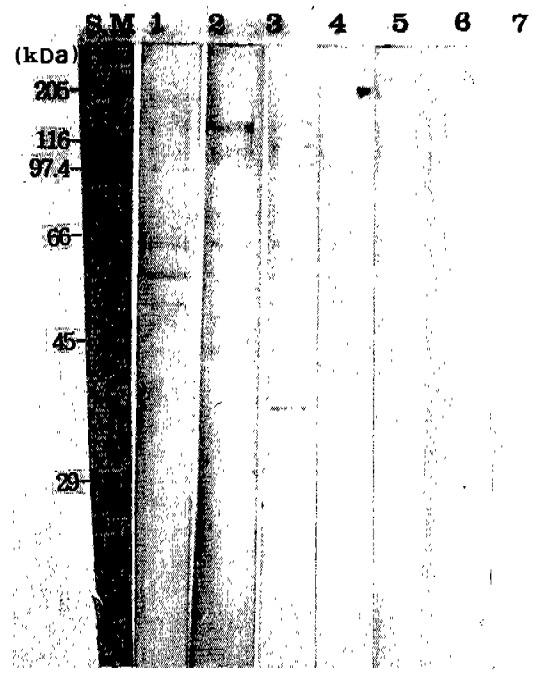

Fig. 6. Immunoblot patterns of serum IgA antibodies to the E64 supplemented crude extracts of *E. dispar*. Lanes 1-3, sera of *E. histolytica* infection; lanes 4-6, sera of *E. dispar* infection; lane 7, control serum.

DISCUSSION

The present immunoblot system reacting human serum to antigenic fractions of *E. histolytica* can differentiate invasive amebiasis. The antigen used for this system was supplemented with E64 on crude extract of axenically cultivated trophozoites of *E. histolytica*. Of 4 proteinase inhibitors supplemented in crude extracts, the E64 supplemented one best preserved high molecular fractions. In the extracts without inhibitors or supplemented with other proteinase inhibitors, the high molecular fractions disappeared and the bottom fraction became thick. This implicated that endogenous cysteine proteinases of *E. histolytica* trophozoites degrade major antigemic fractions and affect the immunoblot pattern. Therefore, the antigen should be prepared in the same way to keep a consistency.

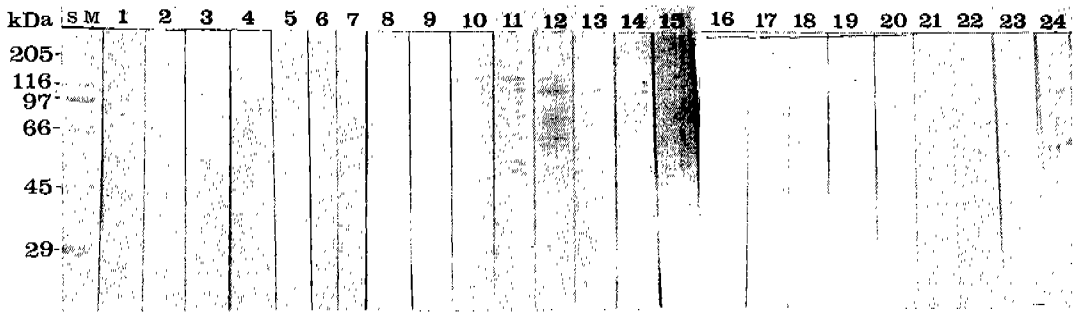

Fig. 7. Screening of 24 symptomless cyst-passers with antigen of *E. histolytica*. The sera probed to the strips 11 and 12 were regarded as infection of *E. histolytica*, and all of ohers were of *E. dispar*.

The antigenic protein bands of the YS-27 *E. histolytica* reacted to the IgG antibody of amebiasis patient sera were 117, 110, 99, 68, 66, 60, 54, 52, 46, and 45 kDa. Contrary to this, all of the antigenic bands of the S16 *E. dispar*, 138, 128, 90, 73, 63, 56, 55, 52, 50, and 43 kDa, reacted to the serum of *E. histolytica* infection which showed a different immunoblot finding from that with *E. histolytica* antigen. The 117 kDa band of *E. histolytica* antigen cross reacted with serum of *E. dispar*, whose functional role is unknown. Anyway, the reaction at the 117 kDa fragment is regarded as non-specific because the sera of negative controls and *E. coli* or *B. hominis* also reacted to the fragment.

As for the immunogenicity of *E. dispar*, it is difficult to identify which bands are from *E. dispar* because the crude antigen was prepared from monoxenically cultivated trophozoites. Fig. 2 displayed that the antigen was a mixture of soluble protein from both *E. dispar* and *Escherichia coli*. However, both sera of *E. histolytica* and *E. dispar* showed no reaction to *E. coli* antigen (data not presented). According to the present findings, anti-*E. histolytica* sera include antibodies to *E. dispar* antigenic fractions as well as anti-*E. dispar* sera. Since *E. dispar* is non-invasive, it must be less antigenic, however, the bands of 90, 63, 55, 50, and 43 kDa of *E. dispar* were found antigenic.

Different sized antigenic fractions have been recorded in different strains of *E. histolytica*. About 20 fragments were found in HK-9 strain and 32 fractions were recorded in HT-31 strain (Chang *et al.* 1979). In NIH:200 strain, major antigenic fractions to the serum IgG antibody were 150, 88, 82, 68, 42, 37, and 9 kDa (Aust-Kettis *et al.*, 1983). In HM1:IMSS strain, the antigenic fractions were 144, 140, 136, 132, 93, 70, and 62 kDa (Ximenez *et al.*, 1993) while 220, 190, 160, 125-129, 96, 75, and 46 kDa fractions were also recorded antigenic in the same strain (Edman *et al.*, 1990). It is difficult to compare directly the present antigenic fragments of YS-27 *E. histolytica* to those of other strains.

According to clinical stages of *E. histolytica* infection, different immunoblot, patterns have been recorded (Ximenez *et al.*, 1993). Cases of active amebic liver abscess frequently showed serum IgG antibodies to 144, 140, 136, 132, 93, 70, and 62 kDa bands while the serum of past amebic liver abscess reacted to the 144 and 140 kDa bands. The cyst carriers showed serum antibodies to 136 and 62 kDa fbands (Ximenez *et al.*, 1993). This clinically related immunoblot patterns partly represent different roles of the antigenic peptides. At least the bands of 132, 93, and 70 kDa are antigenic only in active liver abscess. The present study showed that symptomless cyst-passers of *E. histolytica* produced serum IgG antibodies to 117, 110, 99, 68, 66, 60, 54, and 52 kDa bands while those of clinical colitis cases reacted to all of these 8 fragments together and additionally to the 46 and 45 kDa bands. This point should be evaluated in the future with more cases.

The present study also tried to evaluate the antigenicity of *E. histolytica* and *E. dispar* to IgA, IgM, and IgE antibodies in serum of infected cases. The antigenic bands of *E. histolytica* between 180 and 45 kDa reacted to serum IgA antibody but no differences were

recognized between the reactions to the sera of *E. histolytica* and *E. dispar* infections. When the immunoblot was probed to the serum IgA antibody of *E. coli* or *B. hominis* infection, the reaction pattern was the same with that of *E. histolytica* infection. Furthermore, the present immunoblot system could find no reacting antigenic fractions to the IgM or IgE antibodies in serum of *E. histolytica* infection.

The present immunoblot system was applied to 24 symptomless ameba cyst-passers. Two of them were found to have serum IgG antibodies to 117, 110, 99, 68, 66, 60, 54, and 52 kDa antigen bands of *E. histolytica*. In the remaining 22 cyst passers, the sera reacted to the 117 kDa band only. Although the number of examined cyst-passers was not enough, the present data revealed that more than 90% of symptomless ameba cyst passers are infected with *E. dispar* in Korea. This proportion of non-invasive *E. dispar* infection is a little higher than that of previous analysis of the two species based upon PCR and RFLP (Choe *et al.*, 1996). Unlike the previous study, cultivation of trophozoites in Robinson medium was unsuccessful in the 24 cases of the present study. All of them were clinically normal, but were just found to pass the cyst.

Nowadays, the cyst positive rate of *E. histolytica* is low under 0.1% in general Korean population (Hong, 1994). Based upon the present finding, about 10% of the symptomless cyst passers are regarded as infected by invasive *E. histolytica*. Though it may be hard to draw statistical significance, we roughly presume that one out of 10,000 Korean people may harbour the invasive ameba, *E. histolytica*. The number of infected cases has decreased significantly and continuously but about 4,500 people still spread the cyst into the environment. The risk of ameba infection still exists in Korea, but it is confined mostly to the rural area where human excreta management is incomplete.

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=초록=

면역이적법을 이용한 아질아메바와 동형아메바의 감별진단

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임상증상 없이 검변을 통하여 검출된 이질아메바 포낭양성자를 어떻게 다루어야 할지 오랫동안 논란이 있었으나, 이중에서 병원성이 강한 이질아메바(*E. histolytica*)만 치료하고 병원성이 없는 동형아메바(*E. dispar*)는 치료하지 않는 것으로 의견이 모아져 있다. 따라서 이 두 종을 감별하는 것이 실제적으로 중요하게 되었고 이를 위하여 동위효소 분석, 유전자 분석, 혈청학적인 검사가 가능하다. 그러나 앞의 두 분석법은 원충을 배양하여야 가능하기 때문에 적용하기 어려운 경우가 많아 혈청학적인 감별이 실제적으로 유용하다. 이 연구는 우리 나라에서 무균배양에 성공한 이질아메바 YS-27의 항원성을 확인된 아메바 감염자 혈청과 면역이적법으로 검증하고, 이를 이용하여 무증상 포낭배출자에서 두 종의 감염을 감별하고자 하였다. 시스테인계 단백질분해효소 억제제인 E64를 첨가한 이질아메바의 항원은 117, 110, 99, 68, 66, 60, 54, 52, 46, 45 kDa 분획이 이질아메바 감염이 확인된 사람의 혈청내 IgG 항체와 반응하였고, 동형아메바, 대장아메바, *Blastocystis* 및 대조군의 혈청과는 117 kDa 분획이 미약하게 반응하였다. 포낭양성자의 혈청내 IgA 항체와 많은 분획이 반응하였으나 두 종 양성자간에 차이가 없었고 혈청내 IgM과 IgE 항체와 반응하는 분획이 없었다. 동형아메바의 항원은 두 종 양성자 혈청내 모든 군의 항체와의 반응에서 두종을 감별하지 못하였다. 따라서 이질아메바의 항원과 혈청내 IgG 항체 반응이 두 아메바 감염을 감별할 수 있었다. 증상이 없는 포낭배출자 24명의 혈청을 면역이적 반응시킨 결과 2명에서 46과 45 kDa 분획을 제외한 다른 모든 분획과 반응하여 이질아메바 감염자로 확인하였고 22명은 동형아메바 양성자로 확인하였다. 이 자료를 토대로 하면, 우리 나라에서 검출되는 단순 포낭배출자의 90% 이상은 이질아메바가 아니라 동형아메바 감염자이고, 이에 따라서 포낭양성자 중 환자가 많지 않은 사실을 설명할 수 있을 것이다. 이질아메바 조항원을 이용한 면역이적법은 증상이 없는 아메바 포낭배출자 중 치료를 꼭 필요로 하는 사람을 찾아내는 방법으로 유용함을 확인하였다.

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