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Comparison of the Recombinant Glucosephosphate Isomerase from Different Zymodemes of *Entamoeba histolytica* with Their Natural Counterparts by Isoenzyme Electrophoresis

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Abstract

Entamoeba histolytica is the etiological agent of invasive amoebiasis, the third leading parasitic cause of mortality in the world. Our aim was to find a molecular correlation between a glucosephosphate isomerase zymodeme analyses in *E. histolytica* zymodemes. It was demonstrated that natural and recombinant glucosephosphate isomerase enzymes of *E. histolytica* comigrated in the starch gel electrophoresis, indicating that the isoenzyme pattern of *E. histolytica* glucosephosphate isomerase could be explained from the primary sequences alone and means that expression of the polypeptides of the described sequences in *Escherichia coli* are able to reproduce the classical glucosephosphate isomerase isoenzyme patterns.

Keywords: *Glucosephosphate isomerase,* Entamoeba histolytica*, Zymodemes.*

Introduction

The protozoan parasite *Entamoeba histolytica* is the etiological agent of amoebiasis and a major source of morbidity and mortality worldwide (1, 2). There are an estimated 50 million cases of invasive amebiasis annually (1, 3, 4). This protozoan causes amoebic dysentery, colitis and all forms of extraintestinal amoebiasis such as amebic liver abscess (ALA), and can also asymptomatically be colonized in the large bowel. An estimated 40,000–110,000 people die from amoebiasis annually makes this disease the second leading cause of death from parasitic diseases and has ranked it as the third most important parasitic cause of morbidity and mortality after malaria and shistosomiasis (1-7). At present it is believed that many of the estimated 500 million individuals infected with *Entamoeba* are colonized by non-pathogenic *E. dispar* (6). Colonization of the human gut by *E. histolytica* most commonly results in an asymptomatic infection similar to that of *E.dispar* species (7). Many individuals with *E.histolytica* (in its new definition) infection have no symptoms, and can clear their infection without any signs of the disease. However, 4-10% of asymptomatic individuals infected with *E.histolytica* develop disease over a year (7); where the parasite invades the intestinal epithelium. The reasons for this variability in outcome of infection are, as yet, unclear. However, one possibility is the genetic differences among parasites that may alter the outcome of the disease (8).

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on amebiasis in Mexico emphasized the molecular epidemiological studies should be vigorously pursued to determine whether some subgroups of *E.histolytica* are more likely than others to cause invasive disease (9).

Sargeaunt and co-workers (10) established a method to prove that two distinct organisms, each with different properties, are identifiable by using isoenzyme profiles (zymodemes).

Electrophoretic isoenzyme analysis of several thousand isolates of *E.histolytica* from all over the world by using four glycolytic isoenzymes-hexokinase, glucosephosphate isomerase, phosphoglucomutase, and L-malate NADP+oxidoreductase (malic enzyme)-led to their division into several zymodemes. More significantly, these zymodemes fell into two distinct groups which were re-described as *E.histolytica* and *E. dispar* later (11). The hexokinase (ATP: D-hexose-6-phosphotransferase,EC 2.7.1.1) and phosphoglucomutase (EC 5.4.2.2) isoenzyme patterns are regarded as gold standards for the distinction of *E.histolytica* from *E.dispar* in zymodeme analysis (12), molecular and properties of hexokinase and phosphoglucomutase have been reported earlier (13-17). Moreover, for years, glucosephosphate isomerase (GPI) has been one of the enzymes used for characterization of amebae isolates and it can specially make differentiation between *E.histolytica* strains (zymodemes), however, there are no molecular studies on the *E.histolytica* GPI (10). Zymodeme studies revealed four different stable isoenzyme patterns for *E.histolytica* isolates which are based on the stable expression of α and β bands (18). In order to find the genetically reason for distinct biochemical behavior of *E.histolytica* zymodemes, we examined the *E.histolytica* GPI in four genuine zymodemes.

Materials and Methods

Chemicals and reagents were purchased from Sigma- Aldrich Fine (Tokyo, Japan) or Wako (Tokyo, Japan) unless otherwise mentioned. All other chemicals were commercial products of the highest purity available.

E. histolytica reference strains HM-1: IMSS cl6, SAW1627, SAW755CR clB and KU2 identified as belonging to the pathogenic zymodeme group II, IIα-, XIV and XIX, respectively were used in this study.

Trophozoites of *E.histolytica* strains were axenically cultured in TYI-S-33 medium at 35° C, as described previously (19). Three-day axenically culture of each strain was centrifuged for 4 min at 250×g. The supernatant was discarded and enzyme stabilizers, 1mM final concentration: dithiothreitol (DTT), e-aminocaproic acid and ethylene diaminotetra-acetic acid (EDTA), were added. Freezing at -80° C, followed by thawing at room temperature produced lysis (20). The lysate then was centrifuged at 250×g for 4 min and the supernatant was stored at-80° C.

Electrophoresis In the starch gel electrophoresis (10, 20), the classical method to distinguish *E.histolytica* zymodemes, the similar mass isoenzymes are negatively charged to a different extent, and migrate towards the anode. The experiment was carried out using lysates of 4 zymodemes of *E.histolytica* (II, IIα-, XIV and XIX) and diluted purified recombinant *E.histolytica* glucosephosphate isomerase (EhGPI) proteins: EhGPI 1, EhGPI 2 and EhGPI 3, as well as a mixture of EhGPI and EhGPI 3. These samples were loaded on starch gel.

Electrophoresis was accomplished using starch gel plates and a potential difference of 16Vcm^{-1} for 3 h. Freshly dissolved, air evacuated, hydrolyzed starch was spread across the 220×135 mm plate to a depth of 1.0 mm. When the plates had set, the lysate was soaked up on to 0.5 mm long, 0.5 mm thick white cotton thread. The thread was inserted into the gel in a position previously marked by a "comb" carrying teeth each 0.5 mm long. The loaded plate was placed directly above and in contact with a cooling plate maintained at +8° C in a tank containing 200mM phosphate buffer pH 8.5. This electrode buffer diluted to 15 mM for use in the 8.9% starch gel.

After electrophoresis a mould was placed over the gel to retain the freshly prepared and mixed

coupling substrates which had just previously been added to the molten, but cooled, 1.2% agar. These were then poured over the plate within the mould. The overlay is consist of 100 mM Tris-HCl pH 8.0, 0.5 mM $NADP^+$, 10 mM $MgCl_2$, 1 mM fructose-6-phosphate (as a substrate), 0.5 U/ml of glucose-6-phosphate dehydrogenase, 0.25 mg/ml MMT tetrazolium, and 0.05 mg/ml phenazine methosulphate (PMS).
Whilst the agar was setting (5 min) the plate was placed in an incubator at 37° C for development. After some 30 min when the bands could be easily seen, a photograph was taken for permanent recording (20).

Colorimetric test A serial dilution of three recombinant GPIs enzymes with 200 mM phosphate buffer was prepared. The assay mixture, containing 100 mM Tris-HCl, pH 8.0, 0.5 mM $NADP^+$, 10 mM $MgCl_2$, 1 mM fructose-6-phosphate, 0.5 U/ml of glucose-6-phosphate dehydrogenase, 0.25 mg/ml MMT tetrazolium, and 0.05 mg/ml phenazine methosulphate (PMS), was added to each well. After some minutes, the appearance of green color was considered as an indicator of the GPI enzyme activity.

Results

Fig. 1 shows a logical decrease in the enzyme activity which was synchronized with the enzyme dilution.
The electrophoresed starch gel, after glucosephosphate isomerase-specific staining, showed that all recombinant GPI displayed glucosephosphate isomerase activity (Fig. 2). The EhGPI 3, the isoform of the lowest pI (predicted pI 6.73), migrated faster than EhGPI 1 (predicted pI 6.91) and was found in the same position of β band of zymodeme XIV. Moreover, the band of EhGPI 1 was located at the same place of α band of zymodemes II and IIα-. The EhGPI 2 with the predicted pI of 7.15 demonstrated a band (Fig. 2) more cathodic than the α band in zymodeme II which was similar to the α- band in GPI zymogram of zymodeme II α-. A triple-band comparable with zymodeme XIX was accomplished by mixing EhGPI 1 and EhGPI 3 (Fig. 2).

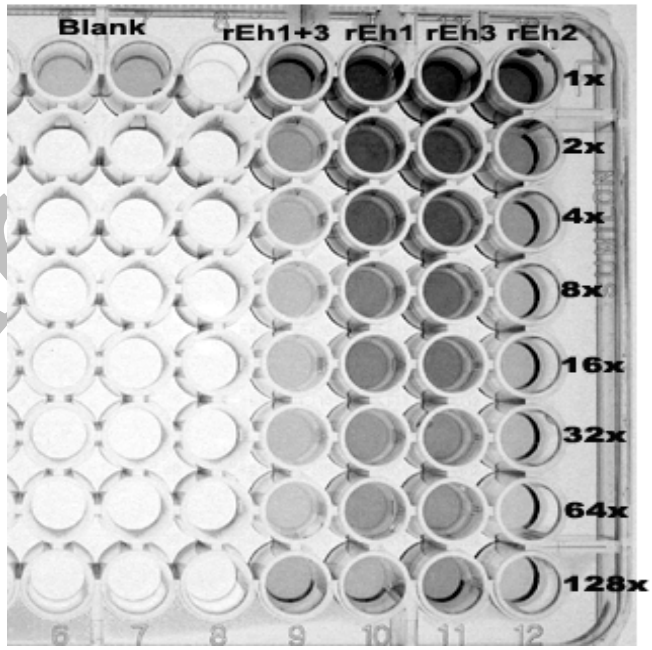

Fig. 1: Colorimetric test of recombinant *E. histolytica* glucosephosphate isomerases. The figure shows correlation of the rEhGPI activity with its concentration. rEh1, recombinant GPI from HM-1: IMSS strain; rEh2, recombinant GPI from SAW1625; rEh3, recombinant GPI from SAW755.

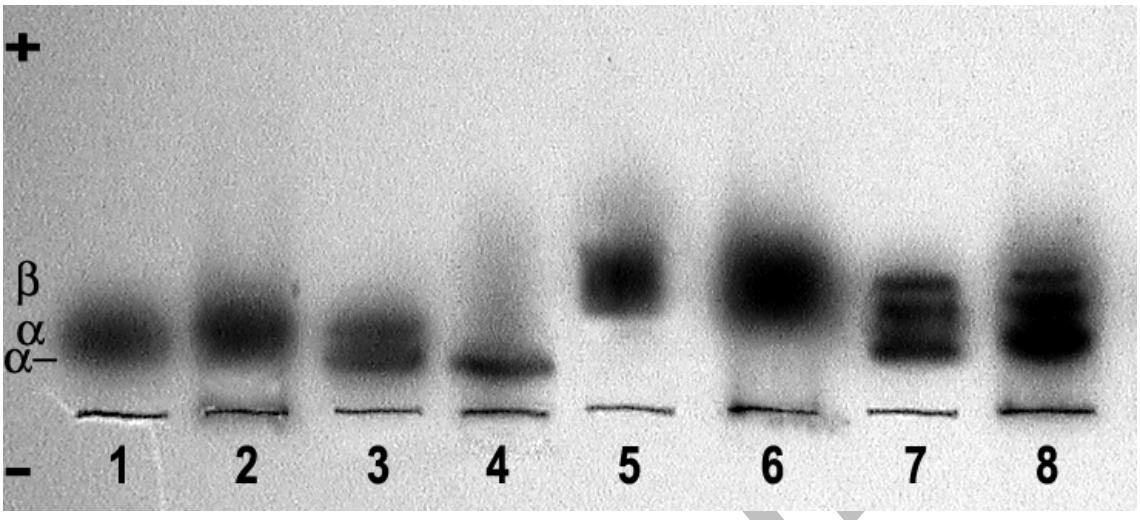

Fig. 2: Photograph showing result of electrophoresis (zymogram) of glucosephosphate isomerase in *E. histolytica* strains compare with their recombinants. Profiles in GPI of: (1) zymodeme II (HM1); (2) rEhGPI 1; (3) zymodeme IIα- (SAW1627); (4) rEhGPI 2; (5) zymodeme XIV(SAW755); (6) rEhGPI 3; (7) zymodeme XIX (KU-2); (8) Mixture of rEhGPI 1 and rEhGPI 3.

Discussion

Cloning, sequencing, functional expression and enzymological characterization of *GPI* gene from the four DNA strains of *E.histolytica* have been described recently (Razmjou, et al. in press). The sequence analysis of achieved clones defined that there are six different alleles of *GPI* gene among four different strains of *E. histolytica*. These alleles differ only in 8 nucleotides which are translated into four different substitutions in protein sequences and leads to expressing of three types of GPI proteins.

The classical gold standard method to distinguish *E. histolytica* subgroups is the examination of the isoenzyme patterns of glucosephosphate isomerase of amebic culture lysates by starch gel electrophoresis. The situation that the migration of the glucosephosphate isomerase was regarded as the unique way for the differentiation of strains of *E.histolytica,* promoted us to investigate the structural basis of the isoenzyme patterns with molecular cloning methods.

Since all glucosephosphate isomerases of *E. histolytica* had very similar molecular masses, we used zymodeme analysis method which was dependent on the charge of the polypeptide for analysis. The comigration of the recombinant EhGPIs with their natural counterparts means that expression of the polypeptides of the described sequences in *E.coli* are able to reproduce the classical GPI isoenzyme patterns as it was shown before for hexokinase and phosphoglucomutase, the two other enzymes used for characterization of ameba by zymodeme analyses (13-17). Our results in *E. histolytica* glucosephosphate isomerase were identical to those carried out earlier (13-17), which indicated the whole isoenzyme pattern from *E.histolytica* and *E. dispar* hexokinase and phosphoglucomutase could be explained from the primary sequences alone.

In the starch gel electrophoresis (Fig.2), all the recombinant polypeptides gave the same pattern as the natural one. We concluded that the diagnostic migration difference between the strains of *E.histolytica* glucosephosphate isomerase α, α- and β bands was caused by differences in genes which had been arisen from small differences in their primary structure. In a recent study (Razmjou, et al., in press), we observed that there were only two alleles encoding GPI in the genome of zymodeme XIX; therefore, the presence of triple-banded GPI might be the result of its production through a

sexual process and zymodeme XIX could be the progeny of zymodeme II and XIV, as Sargeaunt noted earlier (10). Consequently, the existence of triple-banded GPI has been interpreted as a random association of two subunit types encoded by a heterozygous locus whereas the three-banded pattern of heterozygote is typical of a dimeric enzyme which has been observed in Teleost fish (21). These findings imply that a possibly way to establish a new method for molecular epidemiological studies to determine whether some *E.histolytica* zymodemes are more pathogenic than the others. It appears that the described primer combination is likely to be suitable for the differentiation of *E. histolytica* isolates, in a way which is tightly correlated with the gold standard of zymodeme determination, has the potential to be used for studying the molecular epidemiology of *E.histolytica* in an endemic area.

For future molecular epidemiological studies it not only will be important to distinguish between *E.histolytica* and *E.dispar*, but also amongst different isolates of the same species, to evaluate the strains occurring in various geographical areas, and to correlate those strains of *E. histolytica* most associated with the invasive disease. Although the use of in vitro culture plus isoenzyme analysis was the first method to allow a definitive differentiation between the two species (22, 23), current technologies of culture and isoenzyme analysis is not widely available, and is cumbersome and too time-consuming (24). Therefore, improving this traditional but gold standard method to distinguish between various *E.histolytica* strains with help of molecular methods can be used as a novel accessible way to classification of *E. histolytica* in vast molecular epidemiological studies.

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