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ANALYSIS OF SOLUBLE ENTAMOEBA HISTOLYTICA ANTIGEN USING ENZYME-LINKED ELECTROIMMUNOTRANSFER BLOTS

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Abstract. Soluble antigens of ten strains of *E. histolytica* were studied by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme-linked electroimmunotransfer blots (EITB). No relations of immune replicas to virulence, geographical origin and method of cultivation (xenic or axenic culture) were found. Antigens of all ten strains tested precipitated with anti-*E. histolytica* human serum in the area of 30—43 kD. Antigen of HK-9 strain created in this area a characteristic pattern with all sera containing the specific anti-*E. histolytica* antibodies and, therefore, EITB can be used for excluding false positive results in ELISA.

In connection with the widespread use of serological methods in parasitology and with the effort to obtain specific and reproductible results, the problem of the specification and characterization of antigens becomes more topical. The aim is to use a standard antigen giving minimum of the nonspecific reactions and reacting with antisera against various strains of the same species of parasitic organism.

The study of antigenic make-up of amoebae was used in the past for the differentiation of *Entamoeba histolytica* from related species (Sen et al. 1961; Ali Khan and Meerovitch 1968) and for this purpose it has been utilized till now (Giboda et al. 1987) using modern techniques. Attempts to characterize antigenic properties of different strains of *E. histolytica* were carried out as well, especially in order to find antigenic differences among strains of various virulence and geographical origin (Myjak 1970). Many interesting results were achieved by comparing the membrane and soluble fractions of *E. histolytica* antigen (Mathews et al. 1986) and by studying separated soluble *E. histolytica* antigen (Aust Kettis et al. 1983; Myjak et al. 1986). Specific antigenic fractions were studied as well using monoclonal antibodies (Ortiz—Ortiz et al. 1986).

The present study gives the results of analysis of *E. histolytica* antigens of ten strains using various antisera for the detection of the specific antigenic fractions. We use these antigens in serological tests ELISA, CIEP and IHA and our study was aimed to improving the quality of their results.

MATERIALS AND METHODS

1. Antigens. Soluble antigens were prepared from the *E. histolytica* strains described in Table 1. The HK-9 strain was obtained by favour of Dr. P. Myjak of the Institute of Maritime and Tropical Medicine in Gdynia, the strains IP-1, HM-200 and LL by favour of Dr. J. E. Smith of the Institute of Parasitology, McGill University, Montreal and the strain T by favour of Dr. L. M. Gordeeva of the Martsinovski Institute of Tropical Medicine and Parasitology, Moscow. Other *E. histolytica* strains were isolated by us.

The amoebae from the cultures were harvested, washed three times in PBS, centrifuged at 300 g and equal volume of redistilled water was added to the sediment. The trophozoites were disintegrated by repeated freezing-thawing and the lysate centrifuged at 15.000 g. The supernatant was stored at —65 °C till use.

2. Serum samples. The human sera used for the detection of the blots were divided into 3 groups according to the results of ELISA with soluble antigen of HK-9 strain.

a) Positive sera (No 1—6). The specificity of ELISA was proved by a distinct decrease in optical density (OD) after saturation with the specific antigen. The serum 1 originating from a Kampuchean patient with amoebic liver abscess reached the titre of 1/7200 in ELISA, and of 1/640 in IFAT, and precipitated in CIEP. Sera 2 and 3 were provided by favour of Dr. Poljak from Ostrava. The serum 2 obtained from a patient with amoebic liver abscess reacted in ELISA till 1/3600, IFAT 1/256, CIEP positive. The serum 3 was taken 10 months after a successful specific therapy of a patient with amoebic liver abscess; it reacted in ELISA till 1/1800, IFAT 1/640 CIEP positive. The serum 4 is a standard pooled positive serum with the titre of 1/1000 in ELISA. The sera 147 and 153 originated from foreign students (India and Kampuchea) without amoebae in stool and symptoms of amoebiasis, who were serologically positive in screening examination — ELISA 1/1800, CIEP positive.

b) Nonspecifically positive sera (No 7—10) yielded false positive reactions in ELISA, OD did not decrease after the saturation with a specific antigen. The serum 7 contained the specific antibodies against cytomegalovirus. The serum 8 contained specific antibodies against *Escherichia coli* (titre of 1/512). The serum 9 was procured from an apparently healthy Vietnamese and the serum 10 from a patient with acute schistosomiasis.

c) Negative sera (No 11—13). Normal sera were procured from healthy laboratory personnel.

3. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The antigen samples were diluted with the non-reducing buffer (0.125 M TRIS/HCL, pH 6.8, 20% glycerol, 1% SDS and tracking bromphenol blue) to contain 3—5 μg of protein in 10 μl of applied volume and haeted to the boiling point. The PAGE was performed according to the method of Laemli (1970) using 5—15% gradient gel (80× 80× 0.7 mm) without stacking gel in the Gel Electrophoresis Apparatus GE — 2/4 with the power supply ECPS 2000/300 (Pharmacia, Sweden). The electrophoresis ran until the tracking dye reached the bottom of the gel by constant voltage of 150 V. The gels were stained using Coomassie Brilliant Blue R-250 with following silver ($AgNO_3$) staining according to Görg et al. (1985).

4. Enzyme-linked electroimmunotransfer blotting (EITB) (Towbin et al. 1979). The parallel gels were equilibrated in blotting buffer (25 mM TRIS, 192 mM glycine, 20% methanol and 0.04 % SDS) for 15 min. The proteins were transfered to nitrocellulose membrane (Schleicher and Schuell BA 85) in a home-made blotting apparatus with platinum wire flat electrodes placed in the lower chamber of GE-2/4 apparatus which allows the tap water cooling. The blotting was completed after 2 hours at contant voltage of 30 V. The immunostaining procedure was as follows:

a) Blocking the nitrocellulose sheet in 10% horse serum in phosphate buffer saline with 0.05% Tween 20 (PBS/Tween) overnight at 4 °C.
b) Washing in PBS/Tween at intense shaking for 5× 2 min.
c) Incubation in the human immune serum diluted 1/100 in PBS/Tween for 2 hours.
d) Washing as under b).
e) Incubation in the anti-antibody peroxidase conjugate SwAHuIgG/Px (SEVAC — Prague) diluted 1/1000 in PBS/Tween for 1 hour.
f) Washing as under b).
g) Developing of peroxidase acitivity sites using the 0.035% solution of o-diaminobenzidine. 4 HCL (Lachema, Brno) in the 0,1 M TRIS/HCL buffer pH 7.6 with addition of 15 μg of 30% H_2O_2 immediately before use.
h) Stop the color development by washing out the reagents by several changes of distilled water.
i) Drying the blots between four layers of filter paper, photographing and storing between sealed polyethylene foils.

5. ELISA. A simple solid-phase enzyme-linked immunosorbent assay (Lin et al. 1980) was used for the quantification of antibodies. The amoebic soluble antigen was coated on the solid phase polystyrene of the microtitration plates (NOVOGEN). The antigen-antibody complexes were detected by using the conjugate SwAHuIgG/Px and the acetate substrate solution with o-phenylendiamine and H_2O_2. The specificity of the results was confirmed by a parallel titration after saturation of the serum by specific antigen.

RESULTS

The proteins of soluble antigens were separated by PAGE into many protein fractions. The silver-stained gels with PAGE separated antigenic fractions of the strains HK-9 and HM-200 are shown in Pl. I. Many proteins of various molecular weights are distinctly visible particularly in HM-200 antigen. The fractions in the zones of 14.4 kD, 60 kD and 67 kD are dominant, while the fractions in zones of 14,4 and 43 kD predominante in HK-9 antigen.

The scheme of the immune replicas of all antigens tested with the specific anti-amoebic sera is in Fig. 1. The minor fractions creating only very weak bands visible only in wet nitrocellulose are omitted. There are distinct differences between the immune replicas of the single antigens, but the fact that the common antigenic fractions can be found in all of them is important.

The number of fractions in the nitrocellulose blots was expressively lower than in PAGE. The sera did not precipitate at the sites, where the dominant PAGE protein fractions were found. It means that the whole soluble antigen contains a great amount of proteins without antigenic activity and on the contrary, only some minor fractions of the antigen are reactive.

Each of the antigens created in the zone of 30—43 kD at least some of the bands that created in antigen HK-9 (and also T) the characteristic pattern of 5 bands. The weakest was the reaction of the MGE antigen that created only 1 band in this zone. As to the high-molecular zone (over 94 kD), some antigens reacted strongly — e.g., HK-9 — but the bands are hardly distinguishable from the background and create dark spots. Moreover, the immunodominant fractions were absent from this zone in some of the antigens (HM-200). The differences between the single antigens were distinct in the low molecular zone (under 30 kD). Interesting is the band at ca. 17 kD present in 8 out of 10 antigens. There are no common markers differentiating the axenically cultivated strains from the polyxenic ones, the virulent strains from the avirulent ones. Neither the characteristic common markers for the same geographical origin of strains were found out.

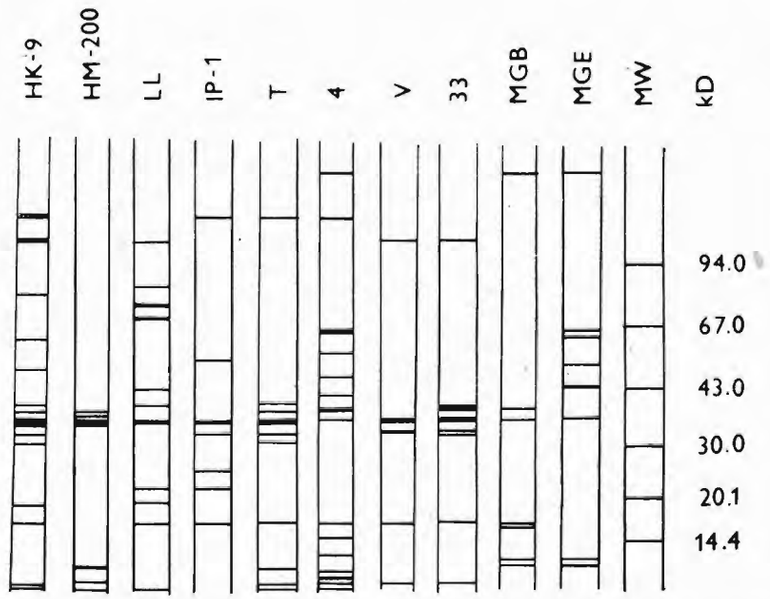

Fig. 1. Comparison of immune replicas of SDS-PAGE of soluble antigens of various *E. histolytica* strains detected by pooled positive anti-*E. histolytica* serum and SwAHuIgG/Px conjugate. Last lane: molecular weight standard proteins.

Using the sera of various patients it has been proved that the sera with specific antibodies against *E. histolytica* yielded a relatively constant pattern in the reaction with the same antigen (Pl. II).

All of these sera precipitated with the HK-9 antigen in the zone of 30—43 kD giving the characteristic pattern of 5 bands. The serum 3 yielded the least distinct reaction, anyway all the 5 bands were slightly visible. As in the preceeding experiment, the sera precipitated as well in the high molecular zone, but the bands are hardly distinguishable from the background. The auxiliary experiments proved that the remains of the culture media (bovine or horse serum) present in the amoebae in spite of washing could interfere in this zone. In the other zones, the precipitation was not regular: e.g. in the low-molecular zone under 14.4 kD the identical precipitates were recorded at the sera 1, 2, 4 and 6 and in the zone of ca. 19 kD at the sera 2 and 6.

The patterns of the sera with false-positive reactions in ELISA were quite different. The serum 8 did not create the precipitates, but dyed the whole background of the nitrocellulose strip (probably nonspecific bond of immunoglobulins at the nitrocellulose). The reaction of the serum 9 was similar. The serum 10 reacted slightly in the high-molecular zone, the precipitate was not distinctly limited. As it follows from Pl. II, the sera negative in ELISA did not react in EITB.

This result was verified on other 40 sera. The pattern of five bands in the specific zone is so constant and characteristic that EITB can be used for the reliable confirmation of serological results in amoebiasis.

DISCUSSION

The soluble *E. histolytica* antigen separated f om disintegrated trophozoites is nowadays used in a considerable part of serological methods. The problem of the serological diagnostics of extraintestinal amoebiasis, when high levels of antibodies present in the patients' sera are detected, can be considered as solved. But the question connected with the detection of low titres of antibodies either persisting after the disease or developing at the subclinical forms of amoebiasis, when the contact of the parasite with the host tissues is limited, has not been solved yet. Also the problem of the proof of the amoebic antigen in the host organism or in stool is topical. The methods detecting antigen traces in the stool were elaborated (Weiss et al. 1987; Grundy et al. 1987) but the antigens used in them are undefined. From the point of view of the improvement of serological methods, the components of amoebic antigen should be separated and defined. One of the methods helping to the characterization of the antigen fractions is EITB. A great advantage of this method is the detection of those antigenic components that take part actually in the serological reactions.

A disadvantage of this and similar methods in the limited possibility to compare the results with those obtained by other authors. The application of the gel gradients is useful for the separation of proteins, but only results obtained in the same gel plate can be compared exactly. The application of protein standards on each plate enable to get more objective results, but one must take into account that the motility of glycoproteins can be different in various gels and the site of the precipitate does not correlate to the molecular weight exactly (Sergrest and Jackson 1972). From this point of view, our results must be compared, e.g., with the analysis of soluble fraction of antigens HK-9, HM-1 and Rahman (Mathews et al. 1986). In spite of some differences in the techniques (Mathews et al. 1986, used 3—25% gel gradient and reached a more detailed separation in the high- and low-molecular zones), it is important that in both studies all strains tested reacted in the zone of 30—43 kD.

This is the reason why we can consider these fractions as a candidate for obtaining a more specific antigen.

Comparison with the results of Aust Kettis et al. (1983) is more difficult, because they did not separate their antigens on the gradient, but used-only 15% acrylamide with 0.16% N,N'-methylenebisacrylamide and for the detection of the blots they used hyperimmune rabbit sera. According to our experience, the results obtained by precipitation with rabbit sera differ distinctly from the precipitates with human sera taken from the persons with amoebiasis.

Table 1. *E. histolytica* strains used for preparation of the antigens

Strain	Way of cultivation	Geographical origin	Form of amoebiasis
HK-9	axenic	Korea	amoebic dysentery
HM-200	axenic	Mexico	amoebic dysentery
LL	axenic	Canada	amoebic dysentery
IP-1	axenic	Carada	cyst carrier
T	polyxenic	Vietnam	amoebic dysentery
4	polyxenic	Vietnam	cyst carrier
V	polyxenic	Brazil	cyst carrier
33	polyxenic	Guinea	cyst carrier
MGB	polyxenic	Afghanistan	cyst carrier
MGE	polyxenic	Vietnam	cyst carrier

One of the intentions of our work was to inquire, if the EITB can be used for the differentiation of virulent *E. histolytica* strains from avirulent ones. Immune replicas of the antigens obtained from the cyst-carriers (Table 1) have no common markers different from the immune replicas of antigens of the isolates from the patients with amoebic dysentery. Comparison of virulent strains with zymodeme II (HK-9, HM-200 and LL) with avirulent strains with zymodeme I (IP-I, MGB and V) did not show any group-characteristic markers. The fact that any relation of antigenic make-up and the geographical origin of the strains were not observed, need not mean that it cannot be found in the future after comparison of antigenic structure of more strains, as was the case with the relation between the geographical origin and zymodemes (Sargeaunt et al. 1984).

Most important of our results is the finding of the zone where all antigens precipitated and the fact that this zone has so typical pattern that it can be used for a reliable differentiation of nonspecifically reacting sera from the sera with the specific antibodies against *E. histolytica*. In this connection a question arises whether it is of advantage to use either the whole *E. histolytica* antigen or its fractions in serological tests. Some authors (Aust Kettis et al. 1983) believe that further purification of any fraction of the amoeba homogenate will not increase the accuracy of the serological tests and that it is useless at the time. Opposite results were presented by Myjak et al. (1986), who proved that the only separation of the soluble *E. histolytica* antigen on sepharose yielded the fractions with distinctly different properties in serological tests. They chose a fraction of lower sensitivity but much higher specificity and they recommended to use the whole antigen for screening and the fraction for the verification of positive results.

We have proved that the SDS-PAGE fractions eluted from the gel were applicable in ELISA; we are now studying this problems in detail. In any case, we are inclined

to the opinion that the more defined antigen, the more accurate the results of serological tests.

АНАЛИЗ РАСТВОРИМОГО АНТИГЕНА *ENTAMOEBA HISTOLYTICA* МЕТОДОМ ЭЛЕКТРОИММУНОБЛОТТИНГА

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Резюме. Изучались растворимые антигены различных штаммов *E. histolytica* с помощью методов ДДС-электрофореза и электроиммуноблоттинга. Не было найдено никаких зависимостей между иммунными картинами и вирулентностью, географическим происхождением и способом культивирования (поликсенические и аксенические культуры). Антигены всех 10 штаммов преципитировали с человеческой сывороткой против *E. histolytica* в области 30—43 кД. Антиген из штамма НК-9 образовал в этой области характеристическую картину со всеми сыворотками, содержащими специфические антитела против *E. histolytica* и поэтому электроиммуноблоттинг может быть использован для исключения ложно положительных результатов полученных с помощью метода ELISA.

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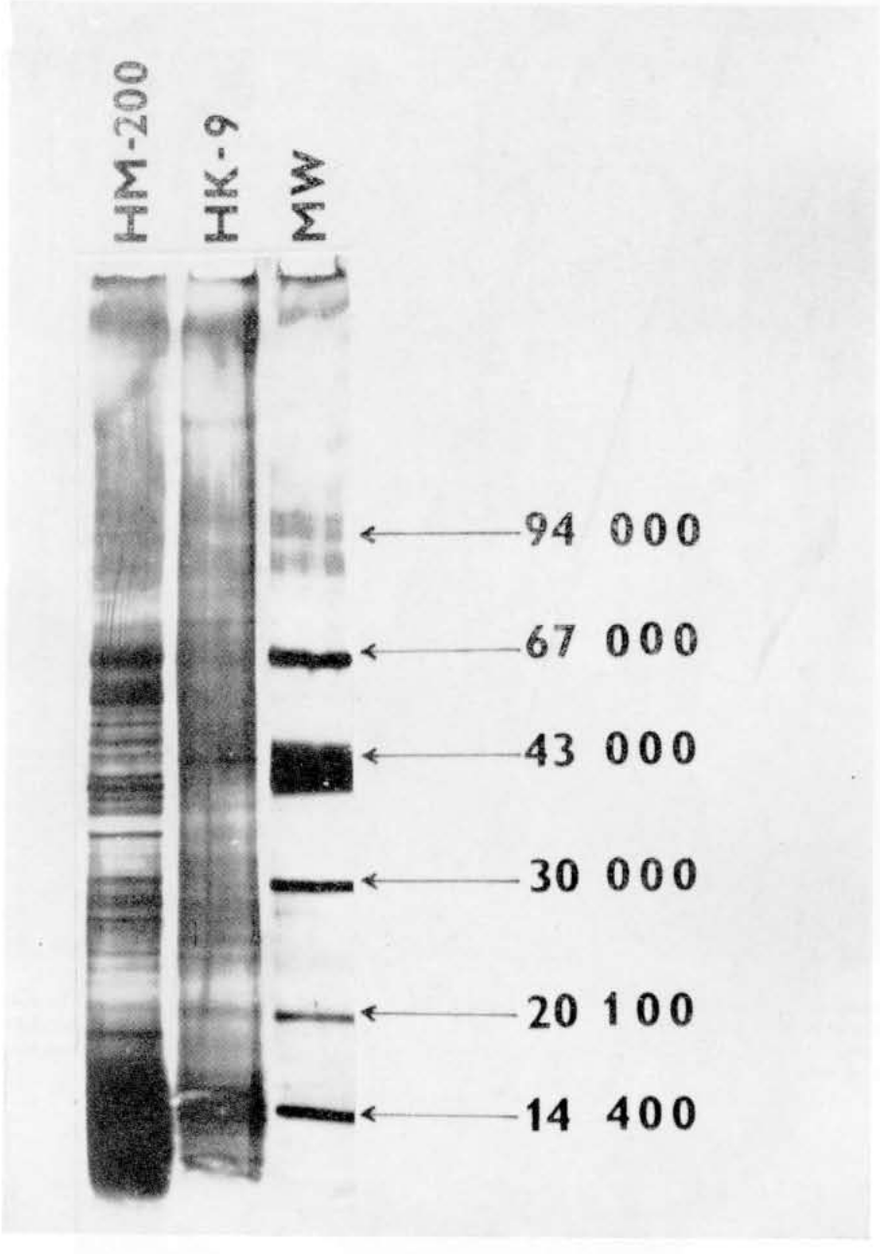
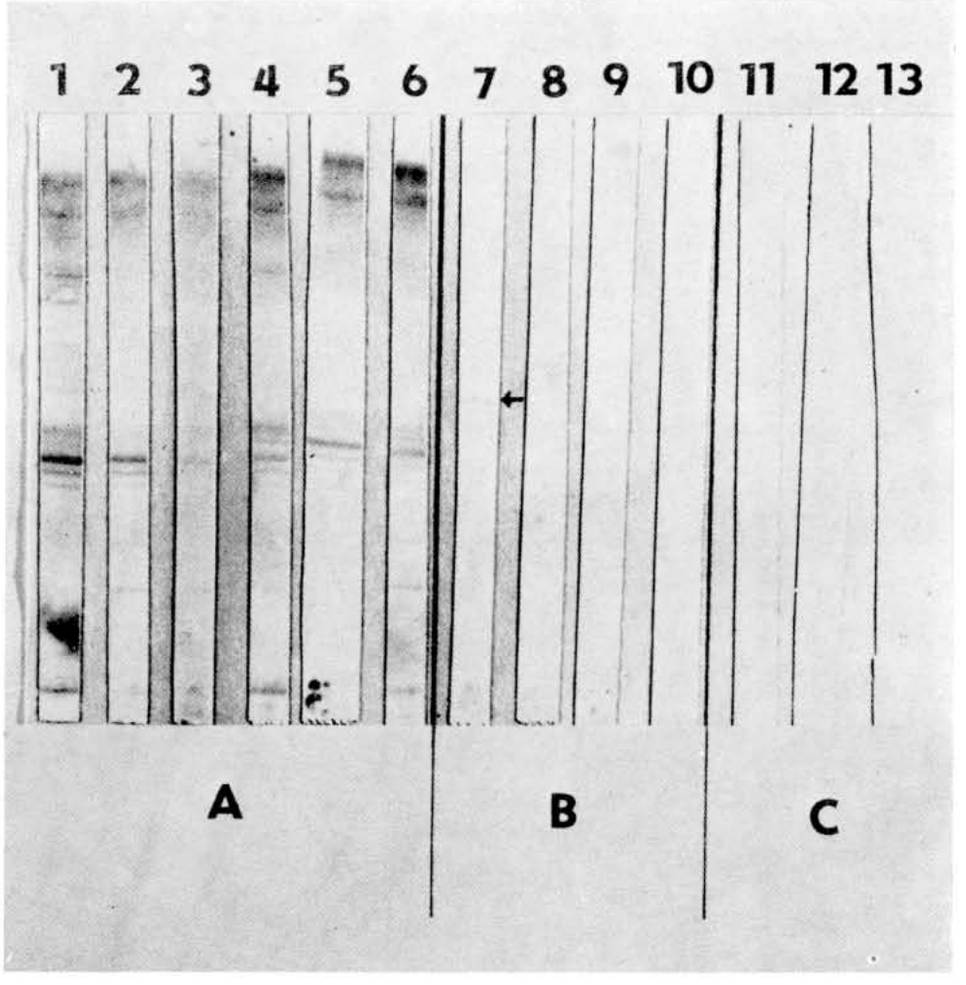


Silver-stained protein profile of *E. histolytica* soluble antigens. Molecular weight of standards in daltons.



Immune replicas of SDS-PAGE of *E. histolytica* HK-9 soluble antigen. Each strip of nitrocellulose sheet was treated with other serum. A: sera positive in ELISA. B: sera falsely positive in ELISA. C: sera negative in ELISA. Conjugate: SwAHuIgG/Px. The arrow shows a band (50 kD) on the strip treated with the serum No. 7.