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Preparation of standard amoeba-antigen from axenic *Entamoeba histolytica* and its use in the serodiagnosis and seroepidemiology of amoebiasis

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Abstract. A method described for large scale cultivation of *Entamoeba histolytica* axenically in a modified Diamond's TP–S–1 monophasic medium. Crude amoeba-antigen prepared by the ultrasonication of the trophozoites of *E. histolytica*, was fractionated by sephadex G–200 column into four different fractions. The whole antigen and its different fractions were freeze-dried and upon reconstitution contained approximately 1·8 mg N/ml or roughly the equivalent of 10×10^6 amoebae per ml. Both whole antigen and its fractions have been used for the detection of specific antibody in the patients' sera. Rabbits were immunised with the antigen and the immunoglobulins were separated from hyperimmune sera by DEAE-cellulose chromatography and salt fractionations. Sera collected from different categories of amoebiasis patients, amoebic liver abscess, amoebic hepatitis, amoebic dysentery, and asymptomatic amoebiasis, were tested serologically using standard amoeba-antigen for serodiagnosis and epidemiological assay of amoebiasis. Results of the assay showed that standard amoeba-antigen is very useful for diagnosis of invasive amoebiasis.

Keywords. Axenic *Entamoeba histolytica*; amoeba-antigen; serodiagnosis; seroepidemiology.

Introduction

The serological diagnosis of amoebiasis, has not so far been possible due to lack of standard amoeba-antigen. Impure antigen prepared from *Entamoeba histolytica*, grown with bacteria, when used for serodiagnosis of amoebiasis patients gave rise to contradictory and variable results. Diamond (1968) first developed an improved method for axenic cultivation of *E. histolytica*, which was later modified by Singh *et al*. (1973). The use of crude amoeba-antigen in commercial kits was discontinued in view of the criticism from the World Health Organisation (Kent, personal communication). Buck *et al*. (1975) reported the criteria for pure. "parasite-antigen" in a memorandum published by W.H.O. In a country like India, where amoebiasis due to *E. histolytica* is endemic (Chuttani, 1968) standard amoeba-antigen can very well serve as an essential reagent for serodiagnosis and epidemiological survey of amoebiasis patients.

This paper deals with preparation of standard amoeba-antigen from *E. histolytica* grown on modified axenic medium, and its use in the detection of specific *E. histolytica* antibody in patients' sera as also epidemiologic survey of amoebiasis patients.

Materials and methods

Axenic cultivation of E. histolytica

E. histolytica strain NIH 200, was obtained from Dr. L. S. Diamond, NIH, U.S.A. The amoebae were grown in Diamonds TP–S–1 medium (Diamond, 1968) which was modified by Singh *et al.* (1973). Instead of ascorbic acid and 0·1 % cysteine, 0·2% cysteine HCl was used. Nine ml medium in a screw-capped tube (16 × 125 mm) was inoculated with 10,000 amoebae/ml of the medium at the bottom of the tube and the tube was incubated at 37° C in upright position without inverting it. The amoebae after 72 h growth were used for the preparation of antigen.

Preparation of amoeba-antigen

For the preparation of crude antigen 100 tubes of axenically grown *E. histolytica* (10^{10} trophozoites) were chilled by dipping in ice-cold water for 10 min to dislodge the amoebae adhering to the glass surface.

The amoebae were collected by centrifugation (at 550 g for 15 min), washed thrice in 50 ml of 0·25 M sucrose and resuspended in phosphate-buffer-saline (PBS) pH 7·2. The suspension was adjusted to a concentration of 10×10^6 amoebae per ml by haemocytometer counts (total yield). The amoeba suspensions were then ultrasonicated for 2–3 min in an ice-cold water bath by use of a Rathion Sonic Oscillator Model DF 101 (10 kc). The preparations were centrifuged at 550 g for 20 min. Aliquot portions (1 ml) equivalent to approximately 1×10^6 amoebae were dispensed into 5 ml screw-capped tubes and frozen in liquid air. The caps of the tubes were slightly loosened and the contents were freeze-dried at 0° C for 16 h and another 8 h at 20° C. The caps of the tubes were tightened, sealed and the tubes stored at 0–4° C.

The protein content was determined according to the method of Lowry *et al.* (1951).

Fractionation of antigen on Sephadex G–200 column

Sephadex G–200 column (1·7 × 25cm) was prepared in 0·1 M sodium-phosphate buffer, pH 7·5, for the fractionation of crude antigen. About 6 mg antigen present in 2 ml buffer was applied on the top of the column. One ml fractions were collected with a flow rate of 15ml/h. Protein under each peak, was pooled and dialysed against several changes of distilled water. Each fraction was freeze-dried and stored frozen.

After preliminary standardisation, the antigen for serological tests was adjusted to 1·8 mg N/ml. Immunoglobulins were isolated by ammonium sulphate fractionation (Stelos, 1967) and DEAE cellulose chromatography (Fahey and Terry, 1967). The antigen-antibody cab reaction was tested by double diffusion, counter-immunoelectrophoresis (Krupp, 1974) Immunoelectrophoresis (Savanat and

Chaicumpa, 1969) and indirect haemagglutination test (Kessel *et al*., 1965 and Prakash *et al*. 1968).

Results

The antigen at 1·8m g N/ml was best suited for serological work. Three well defined peaks (AG_1, AG_2 and AG_4) were observed on Sephadex G–200 chromatography of whole antigen (figure 1). The high molecular weight amoeba antigen came under peak I(AG_1). The heterogeneity of antigen was evident from the multiple precipitin lines obtained in various immunological tests. Hyperimmune serum from immunised rabbit when separated into different fractions (figure 2) on DEAE-cellulose column gave three peaks (HS_1, HS_2 and HS_3). Most of the serum immunoglobulin (IgG) was eluted in the fraction I(HS_1), and gave positive results by gel diffusion test (figure 3).

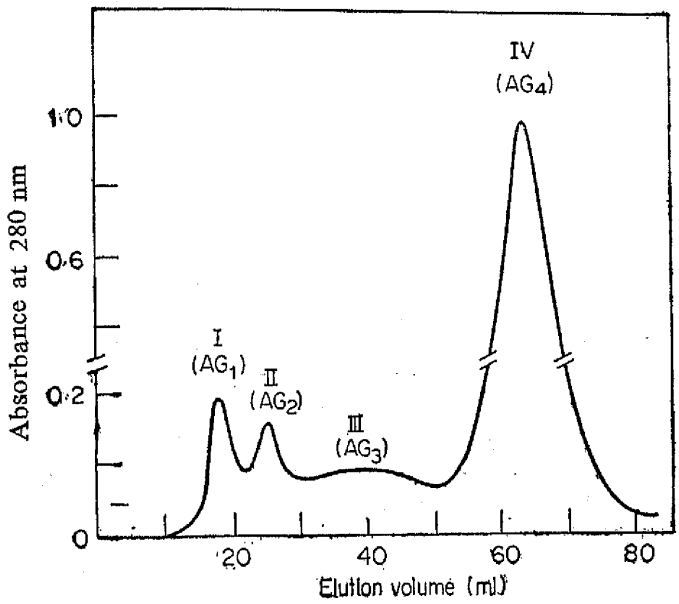

Figure 1. Elution pattern of antigen prepared from axenic *E. histolytica* from Sephadex G–200 column.

Serological tests of 66 sera, collected from different categories of amoebiasis patients were conducted by indirect haemagglutination, counter immunoelectrophoresis, immunoelectrophoresis and gel double diffusion methods using standardised amoeba-antigen. Sera from amoebic liver abscess gave 93·4% positive results by both indirect haemagglutination and counter immunoelectrophoresis tests and 73·4% positive results were obtained by both immunoelectrophoresis and gel double diffusion tests. Sera from amoebic hepatitis cases showed 46·7% positive reaction by counter immunoelectrophoresis and indirect haemagglutination, 33·3% by Immunoelectrophoresis and 26·7% by gel double diffusion tests. In case of acute amoebic dysentery patients, 80% positive results were obtained

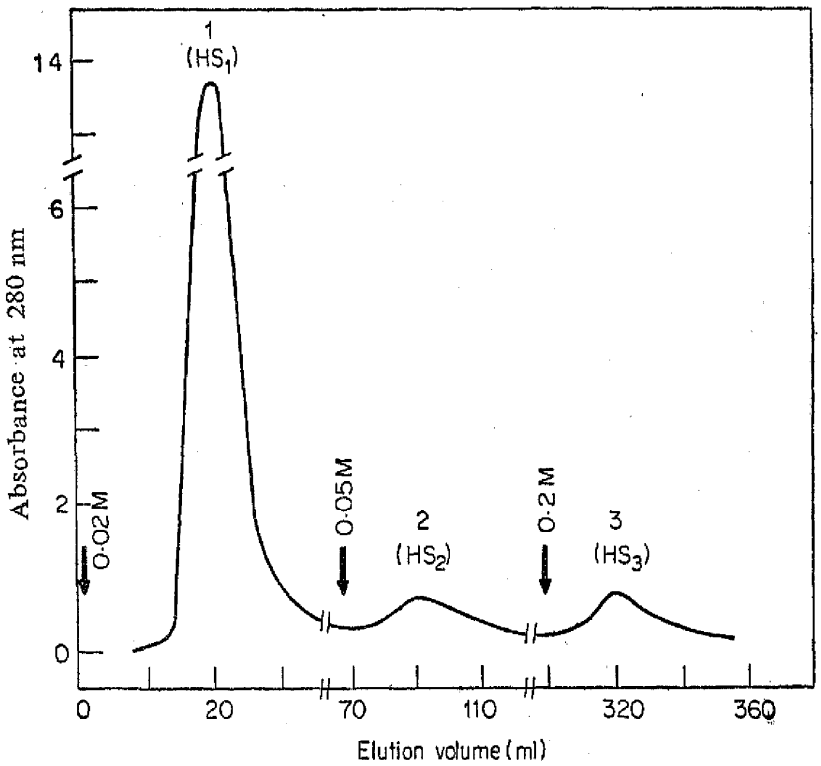

Figure 2. Chromatographic profile of hyperimmune serum of rabbit on DEAE-cellulose.

by all the tests. Asymptomatic patients' sera showed 26·7% positive results by indirect haemagglutination and 6·7 % positive by counter immunoelectrophoresis tests. None of the sera gave positive reaction by immunoelectrophoresis and gel double diffusion tests. Only one serum out of fifteen sera from apparently healthy person showed positive results (6·3%) by indirect haemagglutination test only. No positive results could be obtained by the other three tests (table 1). Among the serological tests conducted, counter immunoelectrophoresis was found to be very simple, rapid and as sensitive as indirect haemagglutination test. Indirect haemagglutination test though very sensitive gave rise to false positive and false negative results. Counter immunoelectrophoresis test was also found to be superior to immuno-electrophoresis and gel double diffusion tests. It is concluded that amoeba-antigen prepared from the axenically-grown *E. histolytica* and purified chemically can be used helpfully for the serodiagnosis and seroepidemiology of amoebiasis.

Alikhan and Meerovitch (1968) reported that IgG and IgM antibodies appeared simultaneously during active immunisation with *E. histolytica* antigens in rabbits and the precipitin was confined exclusively to the IgG class. The IgG haemagglutinin and precipitin constituted the main antiamoebic antibody. IgM haemagglutinin was transitory and decreased gradually. We have also seen that the fraction I of hyperimmune sera of rabbits obtained by DEAE-cellulose chromatography constituted most of the serum IgG and reacted with antigen to produce bands. IgG separated from hyperimmune sera by salt fractionation also, gave similar results, The other two fractions which contained IgA and IgM did not show, any precipitin

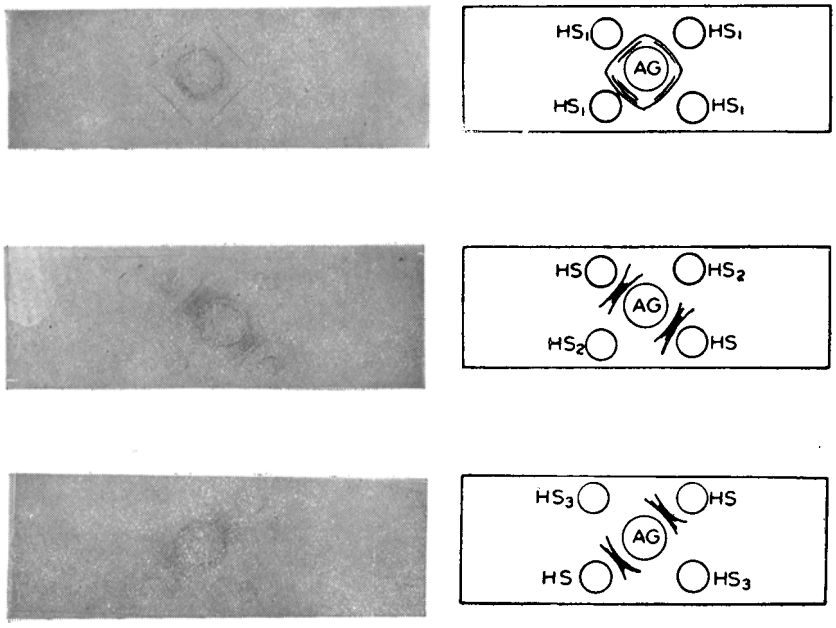

Figure 3. Gel-diffusion test showing reaction of antigen with different fractions of hyperimmune serum (HS_1, HS_2 and HS_3).

Table 1. Serological results with indirect hemagglutination, counter immunoelectrophoresis, immunoelectrophoresis and gel double diffusion tests on 66 sera from human cases of amoebiasis.

Diagnosis	No. of cases	Positive indirect hemagglutination test	Positive counter immuno-electro phoresis-	Positive immuno-electro-phoresis test	Positive gel double diffusion test
Amoebic liver abscess	15	14 (93%)	14 (93%)	11 (73%)	11 (73%)
Amoebic hepatitis	15	7 (46%)	7 (46%)	5 (33%)	4 (26%)
Acute amoebic dysentery	5	4 (80%)	4 (80%)	4 (80%)	4 (80%)
Asymptomatic	15	4 (26%)	1 (6%)	0	0
Apparently healthy	16	1 (6·3%)	0	0	0

band by counter immunoelectrophoresis, immunoelectrophoresis and gel double diffusion tests with, whole antigen. This may be due to use of hyperimmune sera, where immunisation of rabbits with the antigen continued for more than three months, thereby decreasing the level of IgM (Alikhan and Meerovitch, 1968). Human sera from amoebic hepatitis and asymptomatic patients did not show greater positive reaction with the anitigen. This may be due to (i) absence of invasive amoebae; (ii) inability on the part of the patients to produce antibodies; (iii) anti-amoebic treatment received by the patients which might have suppressed the antibody response and poor nutritional status of the patients (Prakash *et al*., 1968).

The application of standard amoeba antigen in the sero diagnosis of various types of amoebiasis patients has clinical applications. Use of impure antigen should never be encouraged in the preparation of vaccine against amoebiasis for developing protective immunity in human subjects.

References

Alikhan, Z. and Meerovitch, E. (1968) *Can. J. Microbiol*., **14,** 1317.

Buck, A. A. (1975) *Bull. WHO*., **52,** 237.

Chuttani, H. K. (1968) *Indian Pract* ., **21,** 670.

Diamond, L. S. (1968) *J. Parasitol* ., **54,** 1047.

Dwyer, D. M. (1972) *J. Protozool* ., **19,** 316.

Fahey, J. L. and Terry, E. W. (1967) in *Handbook of experimental immunology,* ed. D. M. Weir, (Oxford and Edinburgh: Blackwell) p. 19.

Kessel, J. F., Lewis, W. D., Pasquel, C, M. and Turner, J. A. (1965) *Am. J. Trop. Med. Hyg*., **14,** 540

Krupp, I. M. (1974) *Am. J. Trop. Med. Hyg.*, **23,** 27.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951 *J. Biol. Chem*., **193,** 265.
Maddison, S. E., Kagan, I. G. and Elsdon-Dew, R. (1968) *Am. J. Trop. Med. Hyg.*, **17, 540.**
Mahajan, R. C., Agarwal, S. C., Chuttani, A. W. and Chitkara, N. L. (1972) *Indian J. Med. Res* **60,** 547.
Prakash, O., Tandon, B. N., Bhalla, I., Ray, A. K. and Vinayak, V. K. (1968) *Indian Pract*., **21,** 637.
Savanat, T. and Chaicumpa, W. (1969) *Bull. WHO*., **40,** 343.
Singh, B. N., Das, S. R. and Dutta, G. P. (1973) *Curr. Sci*., **42,** 227.
Stelos, P. (1967) in *Handbook of experimental immunology* ed. D. M. Weir (Oxford and Edinburgh: Blackwell) p. 3.
Thompson, P. E., Graedel, S. K., Schneider, C. R., Stucki, W. P. and Gordon, R. M. (1968 *Bull. WHO*., **39,** 349.