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Preparation and Evaluation of Standardized Amoeba Antigen from Axenic Cultures of *Entamoeba histolytica* *

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The successful mass cultivation of Entamoeba histolytica *strains in pure culture has made it possible to produce pure amoeba antigens, which should improve the prospects for immunodiagnostic research and simplify the interpretation of the results obtained in tests for amoeba antibodies.*

The authors have mass-produced immunodiagnostic antigens for amoebiasis serology from 2 axenic strains of E. histolytica *and standardized them as dry powders, which upon reconstitution contained approximately 1.8 mg N/ml, or roughly the equivalent of* 10×10^6 *amoebae per ml.*

The antigens were evaluated with 121 sera from subjects in Costa Rica, South Africa, Taiwan, Thailand and the USA, 49 of the sera being from cases of amoebic liver abscess, 41 from symptomatic intestinal amoebiasis, 19 from asymptomatic intestinal amoebiasis, and 12 from subjects presumed not to have amoebiasis.

Positive results with the indirect haemagglutination test were obtained with 100 % of amoebic liver abscess sera, 90.2 % of those from symptomatic intestinal amoebiasis, 57.9 % of those from asymptomatic intestinal amoebiasis, and 16.7 % of the normal sera. Positive complement-fixation tests for the respective groups were obtained in 83.8 %, 63.3 %, 10.5 % and 0 %, and positive agar-gel diffusion in 79.6 %, 53.7 %, 0 % and 0 %.

These results compare favourably with earlier reports of similar studies in which non-axenic cultures were used and agree well with those of investigators in different parts of the world to whom samples of the axenic antigens were sent.

The authors conclude that the axenic antigens from either strain used, or from the 2 strains pooled, are of broad usefulness in the detection of amoebic antibodies, but point out that the limitations of the tests now used in amoeba serology are not yet clearly understood and that the pattern of antibody persistence after infection requires further study.

The definitive diagnosis of amoebic disease depends upon the unequivocal demonstration of *Entamoeba histolytica* coupled with the presence of the known clinical manifestations of amoebiasis. For numerous well-known reasons, the demonstration and identification of *E. histolytica* may be difficult, with the result that either false-negative or false-positive conclusions may be drawn regarding its presence. Procedures permitting a test for amoeba antibodies should be useful supplements to the above diagnostic measures and should facilitate the epidemiological assessment of amoebiasis in population groups. Much effort and considerable progress in this direction have been made during the past 50 years through the work of numerous investigators using antigens prepared from impure preparations of *E. histolytica*. The lack of pure prepara-

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tions has, however, impeded research on immunodiagnosis and has complicated the interpretation of test results. The successful axenic cultivation of *E. histolytica* by Diamond (1961, 1968) provided for the first time a practical means of producing pure amoeba antigens. This paper deals with various aspects of the preparation, standardization, and evaluation of antigens from axenic cultures.

MATERIALS AND METHODS

Amoeba strains, culture media, and cultivation

Two axenic strains of *Entamoeba histolytica* and methods for their mass cultivation were kindly supplied by Dr L. Diamond (National Institutes of Health, Bethesda, Md., USA). These strains were the 200:NIH, cultured from a patient in the Washington, D.C., area (Tobie, 1949) and the HK9, isolated from a subject in Korea (Geiman & Becker, 1953). The methods and medium for cultivation were slight modifications of those described by Diamond (1968). Briefly, they comprised aseptic cultivation in his monophasic liquid medium (TP-S-1) containing liver digest (Panmede, Paines and Byrne Ltd., Greenford, England), Trypticase (Baltimore Biological Laboratories, Baltimore, Md., USA), glucose, reducing agents (cysteine and ascorbic acid), buffers, horse serum, and a mixture of vitamins. Horse serum proved to be a critical ingredient of the culture medium. Commercially available horse serum varied in suitability from lot to lot, some batches supporting growth poorly or not at all. The screening of horse serum lots was therefore essential for maintaining the cultures.

The cultures were monitored periodically for the presence of contaminants. Examination for PPLO (*Mycoplasma* spp.) was included as an integral part of the preparation of the antigen. Standard procedures were used in testing for PPLO by use of a modification of the medium of Gentry (1960) and as many as 3 serial subcultures before a specimen was to be considered negative.

The strains were maintained in screw-capped tubes (16 mm × 125 mm) containing 15 ml of medium, incubated at an angle of 10° at 36°C. Transfers were made in a sterile cubicle at alternating intervals of 3 and 4 days. Growth was visible macroscopically as a grey deposit.

Mass cultivation was carried out similarly in 125-ml screw-capped Erlenmeyer flasks containing 100 ml of medium and incubated vertically at 37°C. The flasks were inoculated with approximately 1×10^6–2×10^6 amoebae from 3-day-old cultures and the organisms were harvested 3 days later. The average yield per flask (100 ml) was approximately 4×10^6–7×10^6 amoebae.

Preparation of antigen

Large amounts of antigen were prepared from flask cultures of the 200:NIH and HK9 strains of *E. histolytica* in the following manner. The flasks were chilled quickly by immersion for 10 minutes in iced water to dislodge amoebae adhering to the glass. The amoebae were concentrated by centrifugation (at 550 *g* for 10 minutes), washed 3 times in 50 ml of 0.25 M sucrose (as suggested by Mr M. W. Lunde, National Institutes of Health, to reduce lysis), and resuspended in pH 7.2 phosphate-buffered saline. The suspension was adjusted to a concentration of 10×10^6 amoebae/ml on the basis of haemocytometer counts of viable organisms in 2 aliquots. Precautions taken to ensure that the great majority of the amoebae were viable included harvesting during the period of exponential growth (Diamond, 1968) and examination for motility, normal cytology, and reaction to a supravital stain. Bluish eosine was used as the supravital stain in view of the fact that amoebae showing motility or cytoplasmic streaming remained unstained and that earlier work in these laboratories had shown that dead amoebae (heat-killed) stained consistently.

The amoeba suspensions were ultrasonicated for 1–2 minutes in an ice-water bath by use of an MSE ultrasonic disintegrator (20 kHz, 100-W; Measuring and Scientific Equipment Ltd, London, England). Preparations were centrifuged at 550 *g* for 15 minutes to remove the few remaining large particles, which were discarded. The opalescent supernatants were quickly frozen and stored at −20°C to −70°C. Several batches of antigen were thawed, diluted 1:10 with distilled water to facilitate accurate dispensing, and combined into one lot. Amounts of 1 ml, equivalent to approximately 1×10^6 amoebae, were dispensed into vials (filling error less than 1.0%). Fluted rubber stoppers were then inserted partially and the contents were frozen in a freezer at −68°C. Freeze-drying was done in a Del-Vac apparatus (Delvac Engineering Company, Inglewood, Calif., USA; Model 2-2424 RHM Special); the shelf temperature was maintained at 0°C for 16 hours, then raised to 20°C for 8 additional hours, after which the vacuum was filled with dry nitrogen at near atmospheric pressure and the vials were sealed.

Residual moisture content was determined in 2 such lots by the Karl Fischer titration procedure; the values obtained were 2.39% ±0.2% and 4.17% ±0.2%.

Total nitrogen assays were done by the micro-Kjeldahl method. The average values in 4 lyophilized lots following reconstitution ranged from 0.144 mg N to 0.266 mg N per vial.

Standardization of antigen

Standardization of the amount of lyophilized powder per vial was sought in early phases of the work on the basis of the amount of antigen recovered from approximately 1×10^6 amoebae while experience was gained with respect to the total N content of the final preparations. Standardization based on numbers of amoebae had obvious possible sources of error, such as the difficulty of making precise counts due to the clumping of amoebae, variations in their size, and variation in the efficiency of dissolution by brief ultrasonication. Performance tests indicated that adjustment of the undiluted bulk material to 1.8 mg N/ml gave a useful antigen for all 3 serological tests.

Serological tests

Indirect haemagglutination (HA), complement-fixation (CF) and agar-gel diffusion (AG) tests were used to study the antigens. HA tests were done with microtechnique equipment according to the method of Kessel et al. (1965), with 2 differences: (1) erythrocytes from sheep rather than from human subjects were used; and (2) 3% immunoglobulin-free human serum (Grand Island Biological Company, Grand Island, N.Y., USA) or 3% Albuspan, Bio. 864 (25% solution of salt-poor reconstituted human albumin; Parke, Davis and Company) rather than normal human AB serum was used as the stabilizer for cells sensitized with amoeba antigen. Since some lots of the immunoglobulin-free serum gave unsatisfactory stabilization, it was necessary to check each lot before use.

Sera were tested in duplicate or triplicate on separate plates. When parallel tests gave different results, the lower titre was recorded. A control well containing stabilized phosphate-buffered saline, pH 7.2, was employed in each test to ascertain that the sensitized cells did form negative patterns in the absence of antiserum. In addition, each serum was tested at all dilutions for the presence of nonspecific sheep-cell agglutinins using tanned, unadsorbed sheep erythrocytes.

Serum titres were conservatively read as the dilution in the last tube of the series which showed complete agglutination; i.e., an evenly distributed layer of cells covering the entire hemispherical surface of the well. Indistinct rings indicating partial agglutination were not taken into consideration. In the interpretation of results, titres of less than 1:8 were considered as negative, 1:8–1:32 as low, 1:64–1:512 as medium, and greater than 1:512 as high.

The CF test adapted to microtechnique as recommended by the National Communicable Disease Center (Casey, 1965) was followed, except that a haemolysin titration was not performed with each new batch of sheep erythrocytes and that the sera were inactivated at 60°C for 1 hour. Sera were always diluted in duplicate on separate plates and were tested for anticomplementary activity at each dilution.

In the interpretation of results, titres of less than 1:8 were considered as negative, 1:8–1:16 as low, 1:32–1:128 as medium, and greater than 1:128 as high.

AG tests were performed with pre-poured agar slides (Immunoplates, Hyland Laboratories, Los Angeles, Calif., USA) using undiluted serum and reconstituted but undiluted antigen. The wells were filled once with approximately 0.01 ml of test material, and the preparations were incubated at room temperature (24°C). Reactions were read over a period of 2 days with the aid of an illuminated magnifier (Hyland Laboratories).

Titration of antigens

Lyophilized antigen reconstituted to a concentration of 1.8 mg N per ml was adopted for use in AG tests. This decision was made to provide early reactions, easily read bands, and the largest possible number of bands in simple diffusion tests.

Conventional antigen titration studies indicated that the optimal antigen dilution for the HA test was 1:80 with all lots prepared. It can be anticipated that the optimal dilution of antigen may differ with variations in the reagents and procedures used in HA tests.

A decision with regard to an optimal antigen dilution to be used in CF screening-tests proved to be difficult because the optimal dilution varied with the serum used (Table 1). It was apparent from the data that there could be much variation of the optimal antigen dilution within a single test, but that it remained consistent with each serum from

TABLE 1

SELECTED CF OPTIMAL ANTIGEN DILUTIONS FOR A LOT OF 200 : NIH ANTIGEN USED WITH 8 ANTISERA IN 4 CROSS-TITRATIONS

Serum No.	Reciprocal of optimal antigen dilution			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
61 (pulmonary tuberculosis)	8			
43 (amoebic liver abscess)	1 024	2 048	2 048	
54 (amoebic dysentery)			2 048	2 048
44 (amoebic liver abscess)			1 024	
55 (amoebic dysentery)		8		
46 (amoebic liver abscess)			256	256
47 (amoebic liver abscess)	256			
124 (treated amoebic liver abscess)			256	256

test to test. Since sera 3 and 9 were considered negative in the HA test, cross-titration with these were not taken into account in selecting an optimal dilution for the CF test. The conservative decision was made to select 1:128 as the optimal dilution as it seemed that this amount of antigen detected weakly positive as well as strongly positive sera. The antigen was not anticomplementary at any dilution tested.

Sera

Human sera from cases of amoebiasis, as well as from persons suspected of amoebiasis or known to have other disease, were obtained through the courtesy and co-operation of investigators in several widely separated geographical areas, including Costa Rica, South Africa, Taiwan, Thailand, and the USA (see Acknowledgements). The amoebic sera represented asymptomatic infection, symptomatic intestinal amoebiasis, or amoebic liver abscess. The results presented were obtained with sera representing parasitologically confirmed infections or, in the case of amoebic liver abscess in which amoebae could not be demonstrated, infections confirmed by a successful response to specific therapy.

Rabbit sera were collected 1 week following the last of a series of intraperitoneal injections with either strain 200:NIH or HK9 amoeba antigen, which had been lyophilized and reconstituted in distilled water. The immunization was accomplished by giving a series of 8 injections semi-weekly; 1 ml (equivalent to 0.7×10^6 amoebae) was given in each of the first 7 injections and 2 ml (equivalent to 1.4×10^6 amoebae) were given in the last injection.

Salt fractionation of antigen

Antigen of HK9, equivalent to approximately 10×10^6 amoebae/ml, was fractionated by the stepwise addition of saturated ammonium sulfate at 4°C, starting at 10% saturation and proceeding to 20%, 30%, 35%, 45%, 55%, 65%, and 80% saturation. The fractions taken were desalted by gel-filtration on Sephadex G-25 and lyophilized. The initial and final preparations were subsequently assayed in HA and AG tests following reconstitution in the appropriate amount of diluent. The electrophoretic behaviour of the fractions and of the whole antigen was investigated by disc electrophoresis; protein bands were stained with Amido Black.

Sephadex G-200 fractionation of antigen

Antigen of HK9, equivalent to approximately 10×10^6 amoebae/ml, was fractionated by gel-filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden). The fractionation procedure was performed twice, using 5 ml of antigen each time. The column dimensions were 2.5 cm × 85 cm; the eluting solvent was 0.01 M phosphate buffer, pH 7.4; the flow rate was 17.4 ml/h; and absorption at 280 nm was used to monitor the column effluent. The column was eluted with a total volume of 586 ml of buffer, collected in fractions of 1.86 ml/tube (30 drops/tube). At the completion of the run the contents of some of the tubes were pooled to form the fractions indicated in Fig. 1 below. After pooling, the fractions were lyophilized, resuspended in 10 ml of distilled water, and dialysed for 24 hours against 0.01 M phosphate buffer, pH 7.4, and lyophilized again. Before serological testing, each fraction was reconstituted to 5 ml so that the activity of each fraction could be compared with that of the whole antigen.

The nitrogen content of the fractions was determined by the micro-Kjeldahl method, and the carbohydrate content was estimated by the phenolsulfuric acid method of Dubois et al. (1956). The various fractions were compared with each other and with the whole antigen by means of disc electrophoresis, AG, CF, and HA tests.

RESULTS

Developmental studies on antigens

A series of observations were required as guides to the preparation of antigens for practical use.

TABLE 2

NUMBER OF AGAR-GEL BANDS OBTAINED WITH ULTRASONICATED 200:NIH ANTIGEN TESTED AGAINST SELECTED HUMAN SERA [a]

Serum No.	1-minute ultrasonication			2-minute ultrasonication		
	Not centrifuged	Centrifuged supernatant	Sediment	Not centrifuged	Centrifuged supernatant	Sediment
Symptomatic intestinal amoebiasis						
52 (dysentery)	2	2	0	2	2	0
53 (dysentery)	1	1	0	2	2	0
54 (dysentery)	2	(2)	0	2	2	0
55 (dysentery)	(1)	0	0	(1)	(1)	0
122 (acute amoebiasis)	(2)	2	0	2	2	0
123 (acute colitis)	(2)	2	0	(2)	1	0
125 (amoeboma)	1	(1)	0	1	1	0
Amoebic liver abscess						
43	7	5	0	6	5	0
44	3	3	0	3	3	0
45	4	3	0	3	4	0
46	2	2	0	2	2	0
47	2	2	0	2	2	0
124 (treated)	0	(1)	0	0	(1)	0
127	4	4	0	3	3	0

[a] Parentheses indicate a very faint reaction.

Although ultrasonication for 1 minute disrupted amoebae, fewer solid particles remained after ultrasonication for 2 minutes. It was considered desirable to determine whether or not the solid particles contained appreciable amounts of useful antigen. The AG test, which would allow ample time for slow diffusion and reaction, was selected for this purpose. A batch of amoebae was subdivided into 4 portions. Two portions were ultrasonicated for 1 minute and the other 2 for 2 minutes. One portion from each set was tested intact and the others were separated into supernatant and solid phases by centrifugation at 550 *g* for 15 minutes, lyophilized, reconstituted, and tested. The results of tests with 14 reference sera are shown in Table 2. The numbers of positive reactions and of bands agreed well among the intact and supernatant portions separated by centrifugation. No reactions were detected with the sediments. These results led to the decision to discard the sediments in further work.

The production of large amounts of uniform antigen would be simplified if a number of harvests could be made over a period of time and frozen until pooled. It thus became important to know the effects of freezing and thawing on the performance of the antigen. HA tests were used to determine this point. Ultrasonicated 200:NIH antigen was frozen once, twice or 20 times in rapid succession and tested. The results obtained with 3 reference sera are shown in Table 3. No differences were detected among the antigen preparations, which indicated that freezing and thawing before lyophilization were permissible.

The effects of lyophilization were also examined. For this purpose, an aliquot of 1 lot of lyophilized 200:NIH antigen was compared with a similar aliquot of the same lot which had been stored at −20°C for 52 days. The preparations were then examined in HA tests with a reference serum (Table 4). Further similar comparisons were made with 2 other lots of lyophilized 200:NIH antigen by testing 14 sera in AG tests (Table 5) and 9 sera in

HA tests (Table 6). It was apparent from these data that lyophilization did not significantly alter the performance of the antigen.

Stability studies. In the initial absence of information regarding the stability of the antigen, the vials of lyophilized 200:NIH antigen were stoppered in an atmosphere of dry nitrogen and stored, as a routine precaution, at −20°C. The following studies were made on stability relative to temperature. Sealed vials of lyophilized antigen were removed from the freezer and left at room temperature (24°C) for 1–5 weeks. In addition, lyophilized antigen was heated in water-baths at 37°C and 56°C–60°C for intervals of 30 minutes to 8 hours, and at 100°C for 5, 10, and 20 minutes. After heating, the contents of the vials were used in duplicate HA cross-titrations against pooled anti-HK9 rabbit serum. The results at 4 antigen dilutions are given in Table 7. Lyophilized antigen stored at room temperature for 4 weeks, and probably 5 weeks, showed no deterioration. Shelf-life studies were not carried beyond 5 weeks. The data also

TABLE 3

EFFECT OF FREEZING AND THAWING 200 : NIH ANTIGEN ON HA TITRES OF SELECTED HUMAN SERA

Serum No.	Antigen dilution	Serum titre (reciprocal)		
		Antigen frozen and thawed once	Antigen frozen and thawed twice	Antigen frozen and thawed 20 times
54	1:40	4 096	4 096	4 096
	1:80	4 096	4 096	4 096
	1 : 160	1 024	1 024	1 024
44	1:40	16 384	16 384	16 384
	1:80	16 384	16 384	16 384
	1 : 160	4 096	4 096	4 096
46	1:40	4 096	4 096	4 096
	1:80	4 096	4 096	4 096
	1 : 160	1 024	1 024	1 024

TABLE 4

HA CROSS-TITRATIONS USING FROZEN AND LYOPHILIZED ANTIGEN FROM THE SAME LOT OF 200 : NIH ANTIGEN [a]

Antigen dilution	Serum dilution								
	1 : 8	1 : 32	1 : 128	1 : 512	1 : 2T	1 : 8T	1 : 32T	1 : 128T	Diluent control
Serum 47, amoebic liver abscess against frozen antigen									
1 : 40	+	+	+	+	+	±	±	±	±
1 : 80	+	+	+	+	+	±	±	±	±
1 : 160	+	+	+	+	+	±	±	±	±
1 : 320	+	+	+	±	±	0	0	0	±
1 : 640	±	±	±	±	0	0	0	0	0
1 : 1 280	±	±	0	0	0	0	0	0	0
Tanned, unsensitized sheep erythrocytes	±	±	0	0	0	0	0	0	0
Serum 47, amoebic liver abscess against lyophilized antigen									
1 : 40	+	+	+	+	+	±	±	±	±
1 : 80	+	+	+	+	+	±	±	±	±
1 : 160	+	+	+	+	+	±	±	±	±
1 : 320	+	+	+	+	±	0	±	0	0
1 : 640	+	+	±	±	±	0	0	0	0
1 : 1 280	±	±	0	0	0	0	0	0	0

[a] Results expressed as: + = complete agglutination; ± = partial agglutination; 0 = no agglutination.

TABLE 5

NUMBER OF AGAR-GEL BANDS OBTAINED WITH FROZEN OR LYOPHILIZED AMOEBA ANTIGEN TESTED AGAINST SELECTED HUMAN SERA [a]

Serum No.	Frozen (—20°C)		Lyophilized
	200 : NIH for 79 days	HK9 for 58 days	200 : NIH for 20 days
Symptomatic intestinal amoebiasis			
52 (dysentery)	2	2	2
53 (dysentery)	2	2	2
54 (dysentery)	1	2	1
55 (dysentery)	0	0	0
122 (acute amoebiasis)	1	2	1
123 (acute colitis)	1	2	2
125 (amoeboma)	(1)	(1)	(2)
Amoebic liver abscess			
43	3	4	5
44	2	2	3
45	3	3	5
46	2	2	1
47	1	2	1
124 (treated abscess)	(2)	(1)	(1)
127	3	2	4

[a] Parentheses indicate a very faint reaction.

TABLE 6

HA COMPARISONS OF 2 LOTS OF STRAIN 200 : NIH ANTIGEN, ONE LYOPHILIZED AND THE OTHER STORED FROZEN AT -20°C

Serum No.	Reciprocal of serum titre	
	Frozen 200:NIH at 1:80	Lyophilized 200:NIH at 1:80
Experiment 17		
43 (amoebic liver abscess)	4 096	4 096
54 (amoebic dysentery)	1 024	1 024
46 (amoebic liver abscess)	1 024	1 024
Pooled normal sera from laboratory personnel	0	0
Experiment 20		
1[a] (amoebic liver abscess)	4 096	4 096
2[a] (amoebic liver abscess)	16 384	16 384
3[a] (amoebic liver abscess)	16 384	16 384
7 (haemorrhagic amoebic colitis)	1 024	256
7a (haemorrhagic amoebic colitis)	64	64

[a] Sera 1, 2 and 3 represent sera from one patient taken serially at 2-week intervals.

indicated no loss in potency in antigen which had been exposed to 37°C and 56°C–60°C in the lyophilized state for periods up to 8 hours or to 100°C for 5 minutes. Heating the lyophilized antigen to 100°C for 10 minutes reduced the optimal dilution of the antigen from 1: 80 to 1: 40, and similar treatment for 20 minutes produced further deterioration.

Lyophilized antigen stored in the freezer (—20°C) performed as well after 6 months as when freshly prepared.

In the reconstituted state, amoeba antigen could not be stored at 4°C for 73–84 days without a pronounced loss in HA activity; the deterioration may have been due to the growth of contaminants. Boiling the reconstituted antigen completely destroyed HA activity.

These studies supported the tentative recommendation that the antigen be stored routinely in the lyophilized state in the cold. They also suggested that when antigen is left at room temperature for more than 4 or 5 weeks, the optimal dilution should be ascertained by cross-titration before use.

Comparison of strains. The choice of antigen is, of course, of primary importance in serology. Two strains of *E. histolytica* from almost opposite sides of the globe were available in this work. To gain background information on their relative suitability for use in immunodiagnosis, HA tests were performed using strain HK9 and 200:NIH antigens at a 1:80 dilution, singly and pooled. The test sera were 4 reference samples from patients and 5 samples from hyperimmune rabbits. The latter included 2 anti-HK9, 2 anti-200:NIH and pooled anti-HK9 and anti-200:NIH sera. The results from 3 experiments are summarized in Table 8. Although occasional small differences (possibly within experimental error) were seen, the over-all results were remarkably similar.

Further comparisons of HK9 and 200:NIH antigen diluted 1:128 were done by means of CF tests with 3 reference human sera (Table 9). Identical results were obtained with the 2 antigens.

In addition, the reconstituted undiluted antigens were compared in AG tests using 14 reference

TABLE 7

EFFECT OF TEMPERATURE AND TIME ON PERFORMANCE OF LYOPHILIZED 200:NIH ANTIGEN TESTED AGAINST ANTI-HK9 RABBIT SERUM [a]

Exp. No.	Treatment of antigen		Reciprocal of HA titre at antigen dilution of:			
	Time	Temperature	1 : 40	1 : 80	1 : 160	1 : 320
49	1 week	Room	4 096	4 096	1 024	256
49	2 weeks	Room	4 096	4 096	1 024	0
49	3 weeks	Room	Not done	4 096	1 024	256
50	4 weeks	Room	4 096	4 096	1 024	0 [b]
50	5 weeks	Room	4 096	1 024 [c]	1 024	0
42	30 minutes	37°C	4 096	1 024	1 024	256
42	1 hour	37°C	4 096	1 024	1 024	0
42	2 hours	37°C	4 096	4 096	1 024	1 024
43	4 hours	37°C	4 096	4 096	1 024	256
43	8 hours	37°C	4 096	4 096	1 024	256
44	30 minutes	56°C	4 096	4 096	1 024	0
44	1 hour	56°C	4 096	4 096	1 024	0
46	2 hours	56°C	4 096	4 096	1 024	0
46	4 hours	56°C	4 096	4 096	1 024	0
46	8 hours	56°C	4 096	4 096	1 024	0
43	5 minutes	100°C	4 096	4 096	1 024	0
44	10 minutes	100°C	4 096	1 024	256 [d]	0
50	20 minutes	100°C	1 024	1 024	256	0

[a] Fresh antigen samples were not included in these experiments as controls.
[b] The duplicate titre was 1 : 256.
[c] The duplicate titre was 1 : 4 096.
[d] The duplicate titre was 1 : 1 024.

human sera. This experiment also included a comparison of frozen *versus* lyophilized 200:NIH antigen. Remarkably similar results were again obtained (see Table 5).

The foregoing information indicated that the antigens can be used interchangeably and can be pooled if desired. Much of the work of evaluation presented here was performed with an antigen made of approximately equal amounts of 200:NIH and HK9 amoebae.

Salt fractionation studies of HK9 antigen. The stepwise addition of saturated ammonium sulfate, starting at 10% saturation, produced no visible precipitation until 45% saturation was achieved. Four precipitated fractions were made at 45%, 55%, 65% and 80% saturation; the supernatant from the 80% precipitate was taken as the fifth fraction. Serological assays were done with hyperimmune anti-HK9 rabbit serum. The results of HA and AG tests are given in Table 10. All fractions showed some activity in HA tests, with the exception of the supernatant from the 80% fraction. The 55% fraction gave a higher serum titre and could be used at a higher dilution than the whole antigen. Considerable similarity among the fractions was noted in precipitin-line pattern in AG tests, with the exception of the 80% fraction which produced only very weak lines.

In general, relatively poor separations of proteins were attained by disc electrophoresis. It was evident, however, that the major protein-staining band present in the electrophoretic pattern of the whole antigen was the main component in the 55% fraction.

Sephadex G-200 fractionation of HK9 antigen. The elution pattern for the separation of whole

TABLE 8

COMPARISON OF 2 AXENIC STRAINS OF *E. HISTOLYTICA* (200 : NIH [a] AND HK9 [b]) AS INDIRECT HAEMAGGLUTINATION ANTIGENS AGAINST SELECTED SERA

Serum No.	Reciprocal of serum titre [c]		
	HK9 at 1 : 80	200 : NIH at 1 : 80	Pooled HK9 and 200 : NIH at 1 : 80
	Experiment 27		
Rabbit serum 1, anti-HK9	256	256	256
Rabbit serum 2, anti-HK9	1 024	1 024	1 024
Rabbit serum 3, anti-200 : NIH	64	256	64
Rabbit serum 4, anti-200 : NIH	16	64	64
	Experiment 31		
Pooled rabbit anti-HK9 1 and 2 above	4 096	4 096	4 096
44 (amoebic liver abscess)	16 384	16 384	16 384
45 (amoebic liver abscess)	16 384	16 384	16 384
	Experiment 16		
52 (amoebic dysentery)	1 024	1 024	
43 (amoebic liver abscess)	4 096	1 024	

[a] One antigen lot was used in experiments 16 and 17; a different lot was used in experiment 31.
[b] One antigen lot used in all experiments.
[c] Each 4-fold increase in antiserum titre represents one serum dilution.

TABLE 9

COMPARISON OF 2 AXENIC STRAINS OF *E. HISTOLYTICA* (200 : NIH AND HK9) AS COMPLEMENT-FIXATION ANTIGENS AGAINST SELECTED SERA

Serum No.	Reciprocal of serum titre	
	HK9 at 1:128	200:NIH at 1:128
54 (amoebic dysentery)	64	64
46 (amoebic liver abscess)	128	128
47 (amoebic liver abscess)	64	64

antigen on Sephadex G-200, as determined by absorption at 280 nm, is shown in Fig. 1. Only two major 280-nm-absorbing peaks were obtained; a relatively sharp peak, fraction I, and a broader peak, fractions IV and V.

The results of the nitrogen and carbohydrate determinations are given in Table 11.

AG test reactions of the fractions with anti-HK9 pooled rabbit serun are shown in Fig. 2. It is apparent that fractions IV and V were devoid of any precipitin activity. Fractions I, II, and III had one common precipitin line, which coincided with another line common to fractions I and III, as shown by the occurrence of the spurs on the line common to the three reactive fractions.

The disc electrophoretic patterns for fractions I, II, and III, and the whole antigen are presented schematically in Fig. 3. No attempt has been made to indicate in the figure the relative intensities of staining of the protein bands. Since fractions IV and V showed no protein-staining bands they are not included in the figure.

The results of HA tests are shown in Table 12. Fractions IV and V were entirely devoid of HA activity and results with them are therefore omitted from the table. With regard to optimal dilution, none of the fractions was as potent as the whole antigen. At low antigen dilutions, fraction I gave considerably higher serum titres than the whole antigen.

Partial agglutination with diluent alone was common with whole antigen and also occurred with

FIG. 1
ELUTION OF HK9 ANTIGEN FROM SEPHADEX G-200

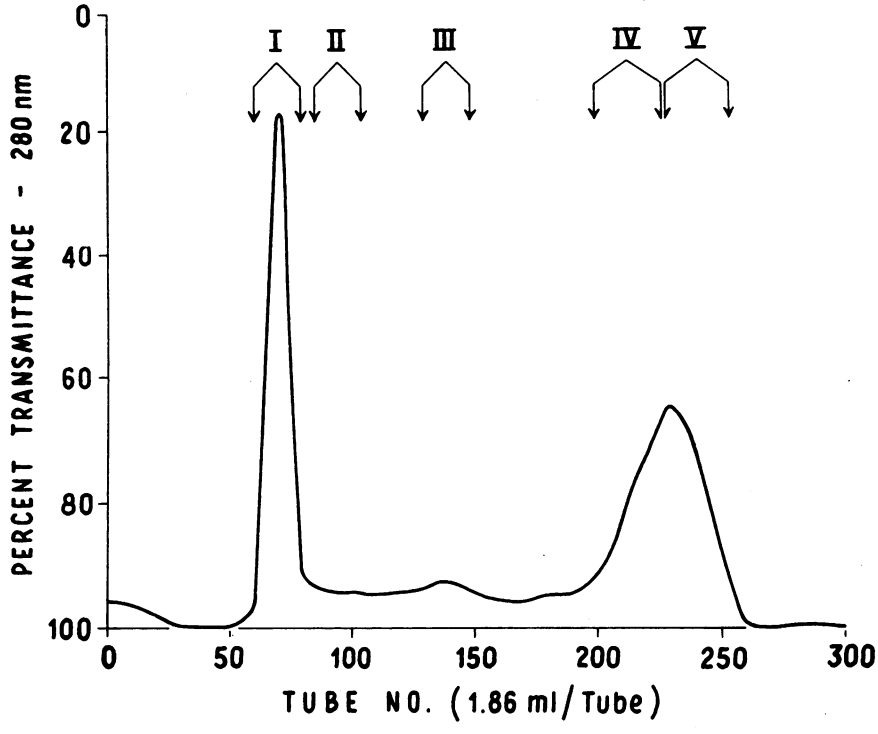
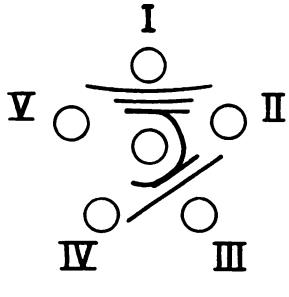


FIG. 2
AG TEST OF SEPHADEX G-200 FRACTIONS OF HK9 ANTIGEN [a]



[a] Centre well: Anti-HK9 rabbit serum. Distance from centre of central well to centres of peripheral wells: 5 mm.

FIG. 3
DISC ELECTROPHORESIS PATTERNS OF WHOLE HK9 ANTIGEN AND SEPHADEX G-200 FRACTIONS OF HK9 ANTIGEN

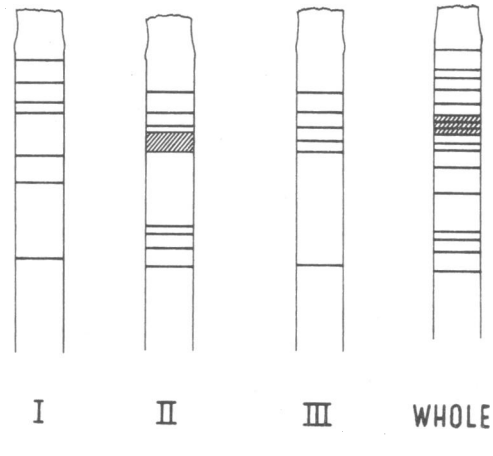


TABLE 10

REACTION OF HK9 ANTIGEN SALT FRACTIONS AGAINST POOLED ANTI-HK9 RABBIT SERUM

Antigen	HA test		Agar-gel (no. of lines)
	Antigen dilution	Reciprocal of serum titre	
Whole (with adjusted solute concentration)	1 : 10	1 024	2 strong, 1 diffuse
45 % saturated $(NH_4)_2SO_4$	1 : 10	1 024	2 medium-strong
	1 : 20	256	
55 % saturated $(NH_4)_2SO_4$	1 : 10	4 096	2 strong
	1 : 20	1 024	
65 % saturated $(NH_4)_2SO_4$	1 : 10	256	2 strong, 1 diffuse
80 % saturated $(NH_4)_2SO_4$	1 : 10	16	2 very faint
Supernatant	1 : 10	0	Not tested

TABLE 11

NITROGEN AND CARBOHYDRATE CONTENTS OF SEPHADEX G-200 FRACTIONS OF HK9 ANTIGEN

Fraction No.	Nitrogen (μg/ml)	Carbohydrate (μg/ml) (as glucose)
I	405	525
II	71	170
III	97	160
IV	67	550
V	20	90

TABLE 12

HA TESTS USING SEPHADEX G-200 FRACTIONS OF HK9 ANTIGEN AND POOLED ANTI-HK9 RABBIT SERUM

HK9 antigen	Result [a] at antigen dilution of:				
	1:10	1:20	1:40	1:80	1:160
Whole	1 024 [b]	1 024 [b]	1 024 [b]	1 024	1 024
Fraction I	65 536 [b]	16 384 [b]	16 384 [b]	4 096	
Fraction II [c]	16 384	4 096	4 096	1 024	
Fraction III [c]	4 096	1 024	256		

[a] Expressed as reciprocal of highest serum dilution at which complete agglutination occurred.
[b] Partial agglutination occurred at all higher serum dilutions and with diluent alone, at this antigen dilution.
[c] No agglutination occurred with diluent alone; partial agglutination was restricted to 2 dilutions or less beyond serum dilution at which complete agglutination occurred.

fraction I; in such cases, partial agglutination likewise occurred at all higher serum dilutions, which required rigid adherence to complete agglutination as the criterion of the end-point. In contrast, fractions II and III did not cause partial agglutination with diluent alone or with the higher serum dilutions beyond the end-point of complete agglutination.

The results of CF tests were not encouraging from the standpoint of the possible benefits of fractionation by this means. Fractions IV and V were devoid of CF activity. Fractions II and III had a very small, almost negligible, amount of activity. Fraction I had approximately one-half the activity shown by the whole unfractionated antigen.

Antigen evaluation

The results of a series of screening-tests using lyophilized antigen are discussed below, first according to the country of origin of the sera and then as a whole. HA tests were performed with 120 sera, CF tests with 96 sera, and AG tests with 121 sera (Tables 13-17). An effort was made to perform and read the screening-tests under uniform conditions. The titres were usually comparable with, but not always the same as, those obtained in the developmental antigen studies. Because there were insufficient amounts of serum in some cases, not all tests were performed with all samples.

Costa Rica (Table 13). All amoebic liver abscess sera from Costa Rica were positive, with high titres in the HA test and with medium to high titres in the CF test. All showed precipitin bands in the AG test.

TABLE 13

SEROLOGICAL RESULTS[a] WITH INDIRECT HAEMAGGLUTINATION (HA), COMPLEMENT-FIXATION (CF) AND AGAR-GEL DIFFUSION (AG) TESTS ON 42 SERA FROM COSTA RICA

Serum No.	HA titre	CF titre	AG (no. of bands)
Amoebic liver abscess			
1	16 384	256	1
2	65 536	512	5
3	16 384	1 024	5
4	16 384	256	3
5	16 384	64	2
6	65 536	32	3
Symptomatic intestinal amoebiasis			
7	1 024	64	1
8	256	32	0
9	64	0	0
10	16 384	64	2
11	4	AC	0
12	16	4	0
13	16	4	0
14	65 536	256	4
15	64	16	0
16	16 384	256	2
17	256	4	0
18	4 096	256	2
19	0	2	0
20	256	2	0
21	4 096	128	1
Asymptomatic intestinal amoebiasis			
22	4	0	0
23	4	2	0
24	256	4	0
25	4	2	0
26	4	8	0
27	16	0	0
28	4	2	0
29	256	4	0
30	64	8	0
31	16	4	0
32	0	0	0
33	16	4	0
34	1 024	0	0
35	0	0	0
36	256	0	0
37	1 024	2	0
38	64	0	0
39	64	0	0
40	0	0	0
Not amoebiasis			
41[b]	16	0	0
42[c]	64	ND	0

[a] AC = anticomplementary; ND = not done.
[b] Ulcerative colitis.
[c] Normal.

The results with 15 sera from cases of symptomatic intestinal amoebiasis were more diffuse. Two of the 15 were negative in the HA test and 6 were negative in the CF test. Only 6 of the 15 were reactive in the AG test and only 4 of these showed strong reactions.

The results with the sera from asymptomatic persons (i.e., carriers) were more interesting because of the occasional positive serum than because of the series of negatives. Although 5 sera in this series (24, 29, 34, 36 and 37) had high HA titres, the CF tests on these were negative. None of the sera in this category was positive in the AG test. The durations of the infections associated with these positive sera were not known.

TABLE 14

SEROLOGICAL RESULTS[a] WITH INDIRECT HAEMAGGLUTINATION (HA), COMPLEMENT-FIXATION (CF) AND AGAR-GEL DIFFUSION (AG) TESTS ON 19 SERA FROM SOUTH AFRICA

Serum No.	HA titre	CF titre	AG (no. of bands)
Amoebic liver abscess			
43	4 096	512	3
44	8 192	1 024	3
45	2 048	128	2
46	1 024	64	1
47	128	32	1
48	1 024	ND	4
49	4 096	ND	2
50	4 096	ND	2
51	4 096	ND	2
Symptomatic intestinal amoebiasis			
52	1 024	4	1
53	512	128	2
54	1 024	32	1
55	32	4	0
56	64	ND	0
57	1 024	ND	1
58	16 384	ND	3
59	1 024	ND	2
Not amoebiasis			
60[b]	0	0	0
61[c]	0	0	0

[a] ND = not done. [b] Arthritis. [c] Pulmonary tuberculosis.

TABLE 15

SEROLOGICAL RESULTS [a] WITH INDIRECT HAEMAGGLUTINATION (HA), COMPLEMENT-FIXATION (CF) AND AGAR-GEL DIFFUSION (AG) TESTS ON 39 SERA FROM TAIWAN

Serum No.	HA titre	CF titre	AG (no. of bands)
Amoebic liver abscess			
62	1 024	4	0
63	4 096	1 024	1
64	1 024	4	0
65	1 024	32	2
66	16	0	0
67	4 096	256	2
68	1 024	4	0
69	4 096	32	2
70	16 384	16	1
71	1 024	16	1
72	4 096	64	1
73	1 024	16	0
74	65 536	512	2
75	4 096	64	1
76	64	AC	0
77	ND	256	2
78	1 024	4	0
79	1 024	1 024	2
80	256	64	0
81	4 096	QNS	1
82	16 384	512	2
83	1 024	32	0
84	1 024	64	1
85	1 024	32	2
Symptomatic intestinal amoebiasis			
86	1 024	16	0
87	1 024	16	0
88	4 096	64	1
89	64	4	0
90	256	16	1
91	1 024	32	1
92	0	0	2
Not amoebiasis			
93 [b]	4	4	0
94 [c]	4	0	0
95 [b]	0	0	0
96 [d]	0	0	0
97 [c]	4	0	0
98 [c]	4	QNS	0
99 [b]	0	0	0
100 [b]	0	0	0

[a] AC = anticomplementary; QNS = quantity not sufficient; ND = not done.
[b] Cholecystitis.
[c] Bacterial liver abscess.
[d] Cholelithiasis.

Two sera from persons reported not to have amoebae were of interest because of low or medium HA titres, although CF and AG results were negative. The significance of these findings must remain open to question since the sera were obtained from persons residing in a heavily endemic area.

South Africa (Table 14). All amoebic liver abscess sera obtained from this country proved to be strong reactors in the HA test, and those for which data were available had medium or high CF titres. All responded with precipitin bands in the AG test.

Two of 8 sera from symptomatic intestinal amoebiasis had a low or medium HA titre; these 2 were also negative in the AG test and 1 was negative in the CF test.

One serum from a case of arthritis and 1 from a case of pulmonary tuberculosis were negative when tested by all 3 methods.

Taiwan (Table 15). Of the 39 sera received from Taiwan, 24 were from clinically diagnosed cases of amoebic liver abscess which were reported to have responded well to antiamoebic therapy. HA tests were done on 23; positive results were recorded in each case, but 1 (66) had a low titre and 2 others (76 and 80) had medium titres. All 24 were tested by CF; 5 were negative and 5 others had high CF titres, while the remaining sera in this category fell into the low-to-medium range. Nine of the sera were negative in the AG test.

Six of 7 sera from symptomatic intestinal amoebiasis cases were HA positive, 2 with medium titres and 4 with high titres; 5 of the 7 were CF-positive and 4 were AG-positive.

None of the 8 sera from cases of other liver disease (bacterial liver abscess, cholecystitis, cholelithiasis) was positive, a finding of particular significance since hepatic amoebiasis had been considered in the differential diagnosis of all of them.

Thailand (Table 16). HA and AG tests were performed on 10 sera from Thailand. All of 7 from cases of amoebic liver abscess had high HA titres. Six of the 7 sera from cases of amoebic liver abscess gave positive AG tests.

One of 3 sera from cases of amoebic colitis was negative in both HA and AG tests, while the other 2 had high HA titres and positive AG reactions.

United States of America (Table 17). Two sera (114 and 115) from a patient in Michigan with amoebic liver abscess had high HA titres and medium CF titres. Both produced clear bands in

TABLE 16

SEROLOGICAL RESULTS WITH INDIRECT HAEMAGGLUTINATION (HA) AND AGAR-GEL DIFFUSION (AG) TESTS ON 10 SERA FROM THAILAND

Serum No.	HA titre	AG (no. of bands)
Amoebic liver abscess		
101	4 096	2
102	16 384	2
103	4 096	1
104	1 024	0
105	1 024	2
106	4 096	1
107	1 024	1
Symptomatic intestinal amoebiasis		
108	4	0
109	4 096	2
110	4 096	1

TABLE 17

SEROLOGICAL RESULTS [a] WITH INDIRECT HEMAGGLUTINATION (HA), COMPLEMENT-FIXATION (CF) AND AGAR-GEL DIFFUSION (AG) TESTS ON 11 SERA FROM THE USA

Serum No.	HA titre	CF titre	AG (no. of bands)
Amoebic liver abscess			
111 [b]	16 384	512	4
112 [c]	4 096	32	1
113 [c]	4 096	32	1
Symptomatic intestinal amoebiasis [b]			
114	1 024	128	2
115	1 024	32	1
116	64	64	0
117	16 384	32	2
118	4 096	ND	2
119	64	ND	0
120	4 096	ND	0
121	256	ND	0

[a] ND = not done.
[b] From Arkansas.
[c] From Michigan, probably acquired in Iraq.

the AG test. This infection was probably acquired in Iraq, although the diagnosis was made in Detroit.

One serum (113) from a case of amoebic liver abscess and 8 sera representing symptomatic intestinal amoebiasis were received from Arkansas. All were positive in the HA test. All of 7 which were tested by CF were also positive, but only 4 of the 8 reacted in the AG test.

Results grouped by disease (Table 18). A summary of the performance of the antigen in the 3 tests is shown in Table 18. The HA test was positive in all of 48 sera (100%) from cases of amoebic liver abscess, 37 of 41 (90.2%) sera from symptomatic intestinal amoebiasis, and 11 of 19 (57.9%) sera from carriers. The CF test was positive in 31 of 37 (83.8%) cases of amoebic liver abscess (the 5 negatives were all from Taiwan), in 9 of 30 (63.3%) sera from symptomatic intestinal amoebic cases and in 2 of 19 (10.5%) sera from carriers. The AG test was positive with 39 of 49 (79.6%) amoebic liver abscess

TABLE 18

RESULTS OF HAEMAGGLUTINATION (HA), COMPLEMENT-FIXATION (CF), AND AGAR-GEL DIFFUSION (AG) TESTS WITH HUMAN AMOEBIC AND OTHER SERA

Diagnosis	HA			CF			AG		
	No. tested	Positive		No. tested	Positive		No. tested	Positive	
		No.	%		No.	%		No.	%
Amoebic liver abscess	48	48	100.0	37 [a]	31	83.8	49	39	79.6
Symptomatic intestinal amoebiasis	41	37	90.2	30 [a]	19	63.3	41	22	53.7
Asymptomatic intestinal amoebiasis	19	11	57.9	19	2	10.5	19	0	0
Not amoebiasis	12	2	16.7	10	0	0	12	0	0

[a] One serum in group anticomplementary.

sera, and with 22 of 41 (53.7%) symptomatic intestinal amoebiasis sera. None of the asymptomatic amoebiasis sera reacted in the AG test. With regard to the small series of non-amoebic sera, all results were negative with the exception of 2 sera in the HA tests; the interpretation of these results is open to question since both sera came from persons residing in a heavily endemic area.

DISCUSSION

These studies have (1) demonstrated the feasibility of producing from 2 strains of axenic *Entamoeba histolytica* useful antigens for the detection of amoeba antibodies by means of indirect haemagglutination, complement-fixation and agar-gel diffusion tests, (2) shown that the antigens can be prepared in reasonably well standardized and relatively stable form as lyophilized powder, and (3) given indications that either strain 200:NIH or HK9 antigens or a mixture of them may be used interchangeably.

Unpublished work in these laboratories has shown that it is feasible to preserve these strains in the frozen state. Furthermore, Schneider & Gordon (1968) have obtained evidence indicating that the components of the medium do not interfere appreciably with the specificity of antigens from cultured amoebae.

The fact that the antigen fraction precipitated by 55% saturated ammonium sulfate showed high serum titres in HA tests and gave satisfactory reactions in AG tests is encouraging. Moreover, in disc electrophoresis this fraction contained most of the major protein-staining band found in the whole antigen. These data suggest that the bulk of the antigenic activity of the whole antigen is located in the 55% fraction and that salt fractionation might be a useful first step in future fractionation attempts.

None of the Sephadex G-200 fractions was as potent as the whole antigen on the basis of optimal dilution for use in HA tests. Fractions II and III had the advantage of avoiding the partial autoagglutination noted with fraction I and not infrequently with whole antigen preparations. Thus, the primary advantage of using this fractionation procedure seemed to be reduction of partial autoagglutination.

Although there was substantial evidence that lyophilization did not damage the whole antigen, its effects on the antigenic potency of the fractions are not clear. Some of the fractions were reconstituted with considerable difficulty after lyophilization, whereas whole lyophilized antigen was easily reconstituted. Further work on fractionation of amoeba antigen utilizing milder methods than lyophilization for concentration of the fractions seems desirable.

The results of HA tests performed with axenic antigen compare favourably with those of Kessel et al. (1965), who used antigens prepared from amoebae grown with trypanosomatids or PPLO. In the present work, all of 48 sera from amoebic liver abscess cases were positive, i.e., had titres of 1: 8 or higher. With the sera from cases of symptomatic intestinal amoebiasis, 37 of 41 (90.2%) were positive, while the sera from the carriers showed 11 positive out of 19 (57.9%). Comparable figures published by Kessel et al. were, respectively, 100%, 98% and 66%.

With regard to CF tests, only 31 of 37 sera (83.8%) from amoebic liver abscess cases were positive. By contrast, Kessel et al. obtained 100% positives with comparable sera. Comparison of our and their figures for symptomatic intestinal amoebiasis (63.3% and 63%) and asymptomatic intestinal amoebiasis (10.5% and 28%) showed essential similarities.

In the present study, the human sera used in the evaluation originated in a number of widely separated geographic areas; there was no difference in the performance of the axenic antigen against these sera, although it may be assumed that antiamoebic antibodies in them developed in response to different strains of amoebae. In this regard, it is recalled that one strain of the axenic amoebae originated in the Washington, D.C., area and the other was isolated in Korea.

A number of investigators in the USA and elsewhere interested in amoeba serology have used samples of the axenic antigen to compare with their own antigens or to commence new programmes with it. Personal communications from these investigators indicate that the axenic antigens were useful in their tests. As examples, R. Elsdon-Dew, in South Africa, compared 2 lots of frozen, but not lyophilized, axenic antigen representing strains 200:NIH and HK9, with his own antigen made of washed amoebae from conventional cultures and reported that the axenic antigen appeared to be more potent than his antigen when used in the AG test; in both cases, the antigen had been adjusted to contain approximately 10×10^6 amoebae per ml.

I. G. Kagan and G. R. Healy, in Atlanta, Ga., USA, found that axenic amoeba antigen made from

strains 200:NIH and HK9 singly and together compared favourably with the DKB+PPLO strain routinely employed by them. W. P. Lewis, in Los Angeles, Calif., USA, found that the results of HA and CF tests with lyophilized axenic amoeba antigen (200:NIH+HK9) compared closely with the results of tests using DKB+PPLO antigen. Encouraging reports have also been received from E. Kotcher in Costa Rica, and J. H. Cross in Taiwan with regard to the usefulness of the antigen when used in the HA test.

A sheep erythrocyte system was used in our HA tests. Since non-specific sheep-cell agglutinins occur with some regularity in human serum, each serum that we tested was accompanied by a control using tanned, unsensitized sheep erythrocytes. In our tests, 23 of 278 human sera (8.3%) showed a titre against tanned, unsensitized cells but this titre was never greater than 1: 4. Accordingly, titres of 1: 4 were not considered positive. This practice agreed with that of Kessel et al. (1965).

Most of the sera were negative when tested against tanned, unsensitized cells. Discounting titres of 1:4 as negative, we decided that a titre of 1:16 was meaningful. An occasional serum with a low titre would therefore be regarded as negative, particularly if serial serum samples from the same patient were unavailable.

The limitations of the tests now used in amoeba serology are not well understood. The HA test was generally the most sensitive, the AG test the least sensitive, with the CF test intermediate. This pattern was occasionally changed (see, for example, the results with sera 26 and 92). The results of one test could not be used to predict the results of the other tests using the same serum sample; this suggested that different antibodies were being measured by the 3 tests. Nevertheless, the axenic antigen appeared to be equally useful in all tests. Other reports have confirmed that the axenic antigen can also be used to advantage in a soluble-antigen–fluorescent-antibody (SAFA) test (Gore & Sadun, 1968), in a bentonite-flocculation test,[1] and a capillary tube precipitin test.[2] The pattern of antibody persistence after infection requires further study. It seems that, in some heavily endemic areas, the likelihood of detecting "residual" antibodies to past infections may be high.[3] This would have to be taken into account before significance could be assigned to titres of individual sera from such areas.

There is so far no evidence to suggest that other infectious agents or disease conditions are responsible for cross-reactions with axenic amoeba antigens. At the same time, however, the work of searching for such evidence using these antigens has hardly begun.

It is too early to estimate the practical advantages of antigens from axenic cultures over others derived from cultures containing microbial associates. It is known that they would avoid the "*welchii* band" noted by Maddison & Elsdon-Dew (1961) in AG tests using antigens prepared from cultures of *E. histolytica* and *Clostridium welchii*. Furthermore, theoretical considerations and general experience in serology favour the use of the purest available antigens. There are, therefore, reasons to give these antigens priority in immediate practical use while research is continued to develop possibly even more useful antigens.

[1] G. R. Healey—personal communication.
[2] S. J. Powell—personal communication.
[3] I. M. Krupp—personal communication.

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RÉSUMÉ

On a préparé, à partir de cultures pures d'*Entamoeba histolytica*, des antigènes normalisés destinés aux épreuves d'immunodiagnostic de l'amibiase.

Deux souches d'amibes, la souche 200 : NIH, isolée aux Etats-Unis, et la souche HK 9, en provenance de Corée, ont été cultivées sur le milieu TP-S-1 de Diamond. Récolté le 3e jour pendant la phase de croissance exponentielle du parasite, le matériel de base a été débarrassé des impuretés non antigéniques, puis remis en suspension dans du soluté salin tamponné et ajusté à la concentration de 10×10^6 amibes par millilitre. Les cultures et les prélèvements ont fait l'objet de contrôles réguliers pour dépister la présence de PPLO (*Mycoplasma* spp.) ou d'autres contaminants. Les amibes ont été ensuite lysées par traitement aux ultrasons, sous réfrigération. Après centrifugation, le surnageant contenant les antigènes solubles a été dilué, mis en ampoules et lyophilisé.

Des essais préliminaires ont montré que le traitement par congélations et décongélations successives, de même que la lyophilisation, n'altérait nullement les propriétés de l'antigène et son rendement lors des épreuves de fixation du complément, d'hémagglutination indirecte et de diffusion en gel de gélose. On a aussi vérifié que l'antigène lyophilisé restait stable pendant au moins 1 mois à la température ambiante et pendant plusieurs mois en cas de conservation à −20°C. Par contre, après reconstitution, la préparation perdait une grande partie de son activité après 73-84 jours de stockage à 4°C.

Les recherches sérologiques menées au moyen de la préparation reconstituée ont permis de définir les conditions optimales d'utilisation de l'antigène: pas de dilution pour l'épreuve de diffusion en gel, dilution à 1 : 80 pour l'épreuve d'hémagglutination, dilution à 1 : 128 pour la réaction de fixation du complément. On a comparé la valeur antigénique des deux souches à l'aide de sérums humains de référence ou de sérums hyperimmuns et constaté que les antigènes de l'une ou l'autre origine pouvaient être utilisés indifféremment ou mélangés.

Par fractionnement de l'antigène HK 9, on a isolé différentes fractions dont l'une contenait la plus grande partie des protéines et l'essentiel de l'activité antigénique. Certaines fractions obtenues sur colonne Sephadex ne présentaient pas le phénomène d'autoagglutination partielle parfois observé avec l'antigène complet.

Les antigènes ont servi à l'examen de 121 sérums prélevés au Costa Rica, en Afrique du Sud, à Taïwan, en Thaïlande et aux Etats-Unis d'Amérique, chez des patients atteints d'abcès hépatique amibien (49 cas), d'amibiase intestinale symptomatique (41 cas) ainsi que chez des porteurs asymptomatiques (19 sujets) et chez 12 personnes considérées comme non parasitées.

L'épreuve d'hémagglutination a donné des résultats positifs dans 100% des cas d'amibiase hépatique, 90,2% des cas d'amibiase intestinale symptomatique, 57,9% des cas d'amibiase latente, ainsi que chez 16,7% des personnes saines. Les taux de positivité correspondants ont été, pour la réaction de fixation du complément, de 83,8%, 63,3%, 10,5% et 0% et pour l'épreuve de diffusion en gel de 79,6%, 53,7%, 0% et 0%. Des résultats concordants ont été obtenus, dans d'autres parties du monde, par des chercheurs utilisant les antigènes normalisés décrits ici.

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