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IMMUNOLOGY OF AMEBIASIS

(Project # 4.227)

Dr. Librado Ortiz-Ortiz

FINAL REPORT

January, 1987

I'm sending you a final report of activities developed in the project "Immunology of Amebiasis", including a group of reprints and manuscripts which are currently either in press or under review.

Throughout the past two years we have been working on the isolation and purification of monoclonal antibodies specific for Entamoeba histolytica. The results of our work have been published (Ximénez et al., 1984; Ortiz-Ortiz et al., 1986). These reports present both the characterization and specificities of these antibodies. We' ve used one of these antibodies for the development of an ELISA test which has turned out to be specific for the determination of E. histolytica antigens in stool samples (Del Muro et al. in press). We are currently field-testing this ELISA, in collaborative work with the National Institute of Pediatrics of Mexico. We have also used these antibodies in the preparation of affinity columns for the isolation of amebic antigens. We have isolated from one of these columns a protein of mw 97000 kd (Fig. 1).

We have also undertaken the antigenic characterization of E. histolytica using several methods. One has been the isolation of mRNA from trophozoites for cell-free translation of amebic antigens (Mendoza et al., 1984), hoping that we could isolate an mRNA which coded for a specific protein and could be useful for recombinant DNA studies. We are currently

starting work on the use of molecular biology for the large-scale production of specific antigens, and these studies are included in our new proposal. We have also continued our work with SDS-PAGE and Western blotting characterizations of the total antigen mixture, with results along the lines presented in our fourth report.

The effect of complement on trophozoites is well-documented, both for the classic and alternate activation pathways (Ortiz-Ortiz et al. 1978; Calderón and Schreiber, 1985). However, we have found that our monoclonal antibodies don't exhibit a cytopathcgenic effect of the type expected from complement activation. On the basis of their high specificity, we decided to attach to them cobra toxin which can activate complement by the alternate pathway. This cobra toxin-monoclonal antibody complex does destroy its amebic target (Vogel et al., 1986), which may be useful for the selective isolation of amebae having specific cell surface determinants.

Previous studies have shown that E. histolytica antigens induce protection against intrahepatic challenge with E. histolytica trophozoites (Sepúlveda and Martínez Palomo, 1984). However, one of the problems we've found with the total antigen is that it precipitates upon storage at 4°C. In order to prevent this precipitation. we found that

the elimination of lipids from this mixture provides chemical stability without altering the protective and immunogenic properties of the mixture (Oliva et al., 1986). We have also found that the total extract of E. histolytica has a strong proteolytic activity and this might also help to explain the unstable nature of our mixture. However, it is interesting to note that when comparative studies on the inhibition of hemagglutination by fresh and stored antigens are carried out, we find the same concentration is required for inhibition whether fresh or stored antigen is used, suggesting that the epitope(s) required for this inhibition is not degraded (Table 1). We are currently trying to characterize the epitope(s) required in this process.

It has been suggested that the ameba evades the humoral immune response by forming polar caps which are then shed or endocytosed (Calderón et al., 1980). On the basis of these findings, we decided to determine whether this phenomenon can be observed in situ, using tissue sections from livers of hamsters with amebic abscesses obtained at different times from the intrahepatic inoculation of E. histolytica. These were stained with: a) FITC-labelled anti-ameba serum, in order to test for antigenic variations, anb b) FITC- labelled anti-hamster globulins in order to show the presence or absence of host globulins on the amebae. Results show that the anti-ameba antibodies react with trophozoites in the hepatic abscess when the anti-ameba

antibody is not found in the blood. If this antibody can be detected in the blood, the number of trophozoites present in the liver which can react with this anti-ameba antibody is slowly reduced until no fluorescence can be detected in the tissue sections. Conversely, the number of trophozoites reacting with the anti-hamster globulin antibody increases gradually, indicating the presence of anti-ameba host globulin on the surface of the trophozoites. We could not detect any evidence of capping in any of the sections studied, indicating this mechanism is not present *in vivo* (Correa *et al.*, 1986).

The immuno-cellular response to amebic infection was studied by measuring the effect of soluble immunomodulators obtained from cell populations in non-immunized animals on the growth of amebae populations *in vitro*. We found that the stimulation of spleen cells with Con A produces soluble mediators which significantly inhibit amebic growth. The growth inhibition mechanism does not seem to be due to lysis by soluble molecules, but rather due to their interference with the cellular metabolism of *E. histolytica*, as is shown by the decreased tritiated thymidine incorporation into DNA and leucine into *de novo* synthesized proteins (Castellanos and Ortiz-Ortiz, 1986). We have found a similar effect by recombinant gamma interferon (submitted for publication).

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FIG. 1

PAGE-Electrophoresis of the fraction obtained by affinity chromatography (A); molecular weight markers (B).

TABLE 1

HEMAGGLUTINATION INHIBITION BY EITHER FRESH OR STORED ENTAMOEBA HISTOLYTICA ANTIGEN[a]

Inhibitor	Hemagglutinating antisera units used: 4	2	1
TOTAL ANTIGEN[b]			
Fresh	+	-	-
Stored	+	-	-
MEMBRANE ANTIGEN[c]			
Fresh	+	-	-
Stored	+	-	-

[a] The different hemagglutinating units of the anti-*E. histolytica* antiserum were incubated with ameba antigen. Afterwards erythrocytes previously sensitized with ameba antigen were added.

[b] Concentration, 250 ng.

[c] Concentration, 500 ng.

IMMUNOLOGY OF AMEBIASIS

(Project # 4.227)

Dr. Librado Ortiz-Ortiz

FOURTH REPORT

August, 1986

Rec'd in SCI: OCT 1 1986

In agreement with the program of activities that we proposed to carry out in the project "Immunology of Amebiasis" and as part of the final report on the work done under the grant, I enclose a series of reprints and of manuscripts which are either "in press" or under review. These describe not only the preparation but also the immunological properties of a monoclonal antibody which is specific for Enatomoeba histolytica (Salud Pública, 1985 and Experimental Parasitology, 1986). This antibody reacts with specific antigenic determinants which are on the membrane surface of E. histolytica and which distinguish it from other Entamoeba species as demonstrated by ELISA and immunofluorescence. The monoclonal antibody also showed sensitivity since it was able to detect as little as one trophozoite or one nanogram of either soluble or membrane amebic antigen. In immunoblot, the monoclonal antibody recognized an antigenic determinant present in only three of the many polypeptides separated by gel electrophoresis of the plasma membrane preparation.

The specificity of the monoclonal antibody was used to develop an immunological test to determine the presence of E histolytica in feces. For this test, after the wells of polystyrene microtiter plates were coated with polyclonal rabbit anti-E. histolytica, preparations of the fecal samples were added to the wells. Thereafter, the specific monoclonal anti-E. histolytica previously conjugated with biotin was added. After the plates were washed, the presence of bound anti-E. histolytica-biotin, which would indicate a positive result, was determined by the sequential addition of streptoavidin-peroxidase and its corresponding substrate and hydrogen peroxide. After a few minutes, the reaction was stopped and the optical density was read in an ELISA reader.

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Initially, 292 fecal samples which had been obtained from the Hospital General del Centro Médico Nacional del IMSS in Mexico City were analyzed in a double blind study by both the traditional coproparasitoscopy and by the ELISA described above. The ELISA gave positive results for all the samples scored as positive by microscopy. In addition, the results of the ELISA showed that the monoclonal anti-*E. histolytica* did not react with fecal samples containing *Entamoeba coli*, *Endolimax nana*, *Giardia lamblia*, *Iodamoeba butschlii*, *Chilomastix mesnili*, *Ascaris lumbricoides*, or *Trichuris trichura*. That this monoclonal anti-*E. histolytica* did not react with *E. coli* must be stressed since *E. histolytica* and *E. coli* are often confused, even by the most experienced microscopist. Twenty additional samples, negative by microscopy, were positive in the ELISA. However, it was impossible to determine if these were false positives or a reflection of the increased sensitivity of ELISA over microscopy since, due to the severe earthquake which hit Mexico City in September 1985 and which destroyed the Centro Médico Nacional, it was impossible to consult the clinical histories of these patients.

In a later study in which 204 fecal samples obtained from the Instituto Nacional de Pediatría in Mexico City were tested by the same double blind analysis, the ELISA gave positive results not only for those samples scored positive by coproparasitoscopy (8/192) but also for an additional 12 samples. When the corresponding cases were reviewed, it was found that the clinical histories of these patients were compatible with a presumptive diagnosis of intestinal amebiasis. The manuscript in which these results were described was submitted to the Journal of Clinical Laboratory Analysis and after some modifications are made will be considered for publication.

We found that several of the monoclonal anti-E. histolytica which we produced do not activate the classical complement pathway. With the aim of exploiting the specificity of these monoclonal antibodies (non-activator of complement) in the elimination of trophozoites of E. histolytica, we prepared a complex of the specific monoclonal anti-E. histolytica and cobra venom factor, an activator of the alternative complement pathway. The resulting conjugate, in the presence of complement, inhibited the growth of trophozoites of E. histolytica in vitro (Arch. Invest. Med., México, in press). Such complexes could be useful in the selective elimination of trophozoites that present the corresponding epitopes, thereby permitting the selection of amebas bearing different antigenic determinats.

To learn more about the immune response in invasive amebiasis, histological studies were carried out in which fluorescein-conjugated rabbit IgG anti-hamster IgG or the monoclonal antibody, were used to determine if the antibodies present in hamsters with amebic liver abscess (ALA) reacted with trophozoites located in the liver. This study is particularly important since it has been reported that the reaction of such antibodies with E. histolytica in vitro induces capping, a phenomenon wich has been postulated as a mechanism by which the parasite may evade the immune response of the host. The results of our experiments indicated that, after hamsters had been intrahepatically inoculated with 3.5×10^4 viable trophozoites, specific serum antibodies were found 15 days post-inoculation, the presence of which correlated with the deposit of antibodies on the surface of the amebae in the hepatic abscess as shown by the histological sections. In the

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presence of host antibodies, the specific monoclonal anti-E. histolytica either did not react, or did so in low proportions, with the amebas in the ALA, indicating that the antigenic determinants of the amebas has been blocked by the host antibodies. Normal liver sections did not react with any of the labelled antibodies. In some cases in which destruction of the amebas was observed, a marked fluorescence was visualized between monoclonal or polyclonal antibody and the cellular detritus, whereas whole amebas present in the zone did not fluoresce (Arch. Inves. Méd. Méx. in press).

We have also continued studying the cellular immunity, in particular, the effect of lymphocytes on trophozoites of E.histolytica. The findings constitute the first report indicating that soluble mediators from non-immune lymphoid populations can cause inhibition of growth of a protozoan parasite. The soluble mediators were obtained from Con A-activated rat spleen cells. A culture system was adapted to assay the Con A-activated supernatants; an incubation period of 48 to 72 h was required to cause significant growth inhibition of amebae trophozoites. Appropriate controls confirmed that this inhibition of ameba growth was not due to depletion of nutrients in the medium. The mechanism of inhibition of growth did not appear to be the result of lysis by the soluble mediators but rather of interference with the cellular metabolism of E. histolytica, as evidenced by the altered incorporation of ^{3}H-thymidine into DNA and tritiated leucine into protein synthesized de novo (Arch. Invest. Méd. Méx., in press).

With respect to immunoprophylaxis, it was demonstrated that total antigen from E. histolytica was immunogenic in various species and, in addition, protected Syrian golden hamsters against intrahepatic challenge

with live E. histolytica (Tables I-II). However, this preparation is unstable when stored in solution at 4°C, due principally to the enzymes present in the extract, thereby limiting its usefulness as a potential vaccine. We found that, when the extract was delipidized, no material was precipitated and the resulting preparation retained its ability to protect animals after intrahepatic challenge with viable E. histolytica (Arch. Invest. Méd. Méx., in press). We are at present continuing these studies and testing other preparations, for example ameba membranes, to determine their protective effect in this experimental model.

To characterize the ameba antigen, we have carried out translation of mRNA isolated from E. histolytica in a cell-free system. The translation products reacted not only with sera from patients with invasive amebiasis but also with monoclonal anti-E. histolytica. The human sera revealed several bands; the monoclonal only a few and three in particular. Translation of RNA in this system offers the possibility of isolating the mRNA of proteins which may be important either in protection or in diagnosis. Such a technique would facilitate the establishment of a more complete gene bank (Salud Publica, 1985). We are also studing the total ameba antigen by electrophoresis in polyacrylamide gel and by immunoblots. We have carried out the following:

1. Total extract of E. histolytica were subjected to electrophoresis in SDS-polyacrylamide gels, under reducing and non-reducing conditions, to study the electrophoretic patterns of the extracts.

2. The electrophoretic patterns of the extract of pathogenic strains of E. histolytica HM1:IMSS were compared with those of extracts of a less virulent strain, HM2:IMSS.

3. The effect of delipidization on the total extract of E. histolytica as determined by the electrophoretic band patterns was studied. (Run under both reducing and non-reducing conditions.)

4. After the electrophoretic band patterns of strains HM1:IMSS and HM2:IMSS had been established, the polypeptides separated by electrophoresis in SDS-polyacrylamide gels were transferred to nitrocellulose sheets. Immunoblots were run with immune anti-E. histolytica (HM1:IMSS) sera, monoclonal IgM anti-E. histolytica HM1:IMSS, normal mouse serum, and other controls.

5. With the aim of isolating the specific antigen(s) of E. histolytica that reacted with the monoclonal antibody, total extract of E. histoytica was separated by affinity chromatography on Sepharose 4B coupled to the monoclonal IgM antibody. The bound antigens were eluted with 50 mM glycine buffer (pH 9.3) and 50 mM diethylamine (pH 11.5).

6. A new cell fusion was carried out to obtain additional monoclonal anti-E. histolytica such that those with higher affinity for the specific antigen(s) of E. histolytica HM1:IMSS may be selected.

At the present time, we are actively working on the last two points, immunoprophylaxis and characterization of the E. histolytica antigen(s) that may be important in protection against amebiasis. We have asked for a renewal of the grant for another two years, in which period we feel we can complete the proposed work.

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TABLE I

IMMUNIZATION OF ADULT HAMSTERS WITH TOTAL ANTIGEN OF *E. histolytica*

Animals per group	Immunogen	Amebic abscess of the liver[a]	Serum antibodies[b]
15	Ameba antigen (1.5 mg) in FCA[c]	4	15
10	Ameba antigen (1.5 mg) alone	3	8
20	FCA	16	7
20	Axenic medium	20	5

[a]The determination of amebic abscess of the liver was done by macroscopic and histologic examination of the liver.

[b]Antibody determination was performed by counterimmunoelectrophoresis 7 days after challenge.

[c]FCA: Freund's complete adjuvant.

TABLE II

IMMUNIZATION OF NEONATE HAMSTERS WITH SOLUBLE EXTRACT OF *E. histolytica*

Immunogen	Amebic abscess of the liver[a]	Serum antibodies[b]
Soluble antigen(2.4 mg)[c]	1/30	30/30
None	30/30	4/30

[a;b]See legend in Table I

[c]Immunization was performed by administering 800 ug of the antigen intraperitoneally on dyas 7, 10 and 13 after birth. A week later, the animals were challenged with 5×10^4 viable trophozoites.

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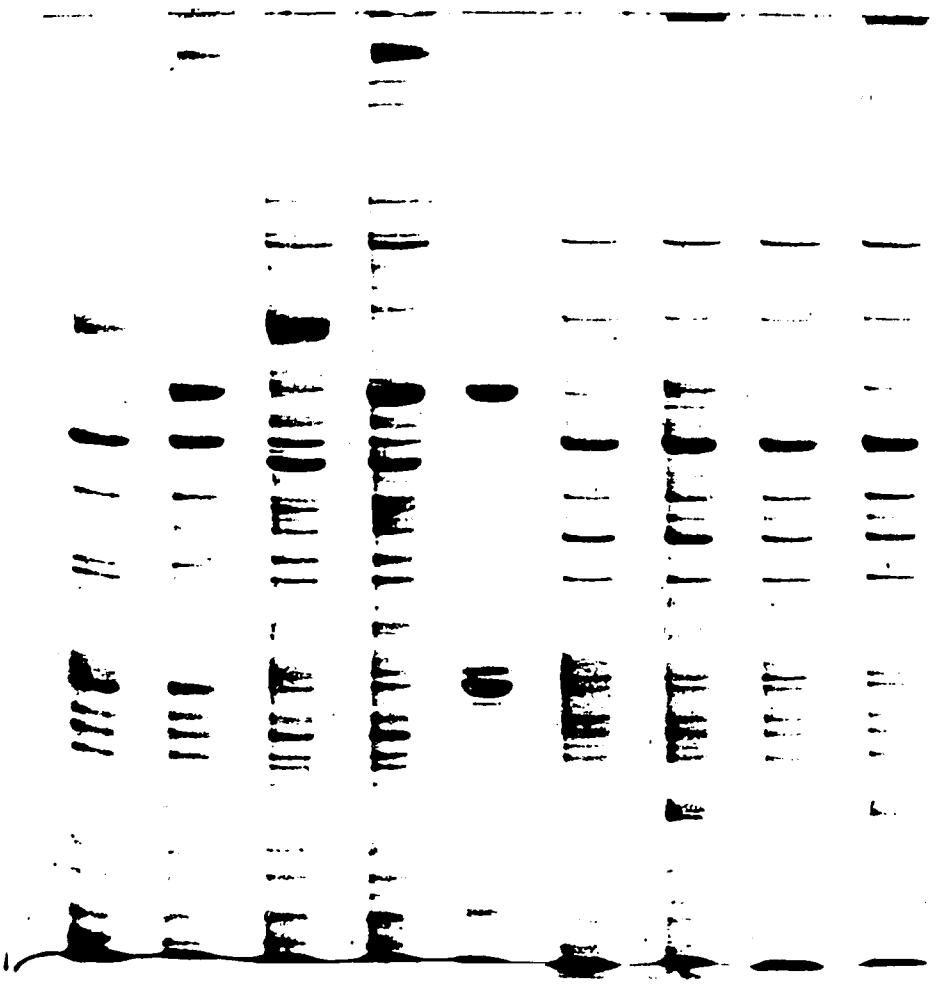
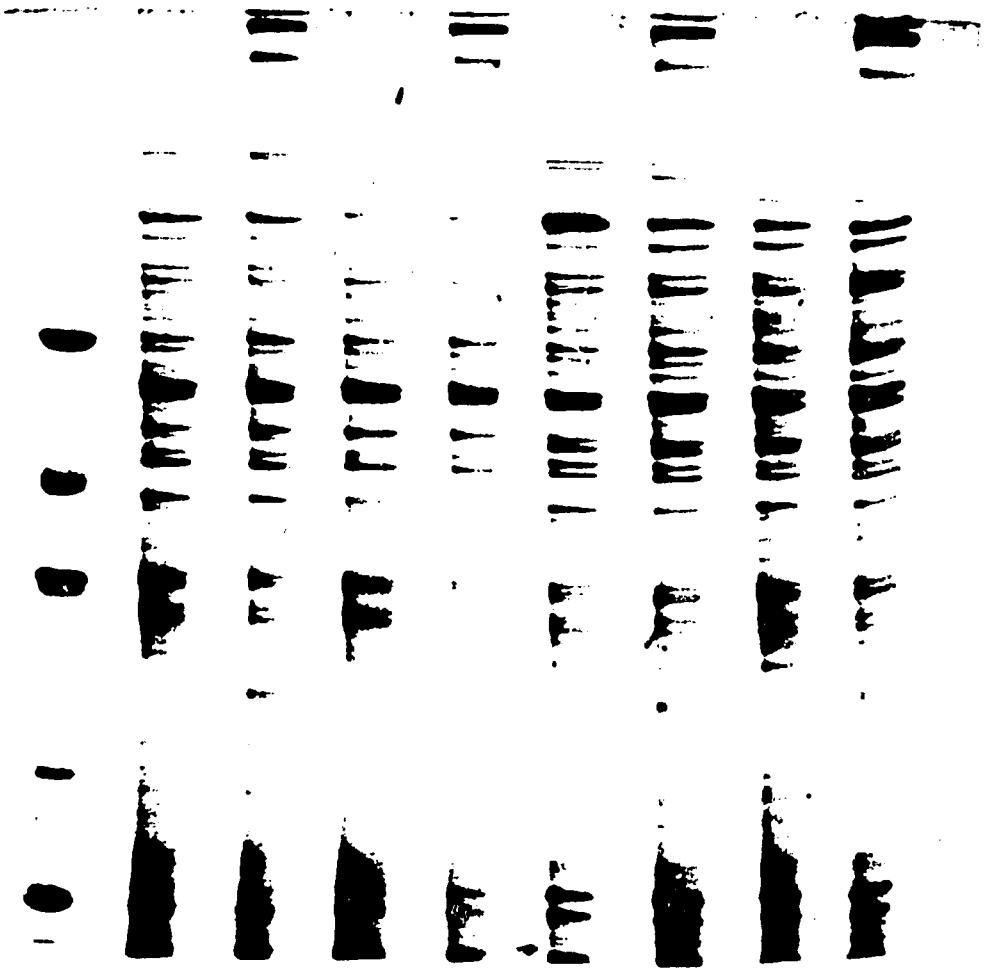


Electrophoresis in 10% acrylamide gel.

Total HM1:IMSS extract (track #1, reducing conditions; #2 Non-reducing conditions); Molecular weight markers (track #5, phosphorylase B, 94,000; albumin, 67,000; ovalbumin, 43,000; carbonic anydrase, 30,000; trypsin inhibitor, 20,100; lactoalbumin, 14,400); Total HM2:IMSS extract (track #6 and 8, reducing conditions; #7 and 9, non-reducing conditions).

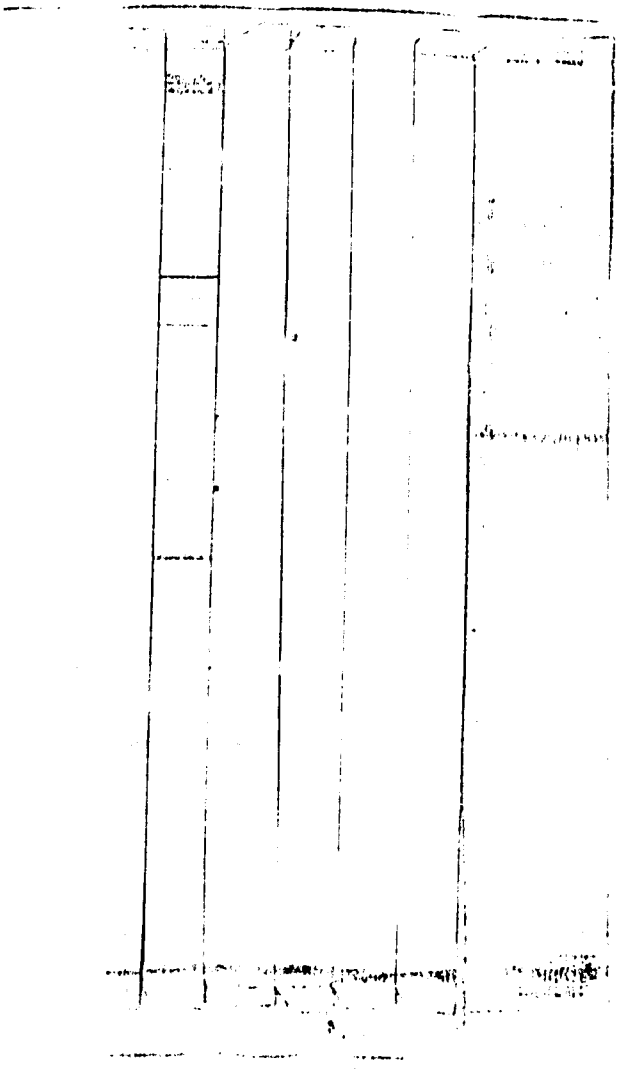


Electrophoresis in 10% acrylamide gel.

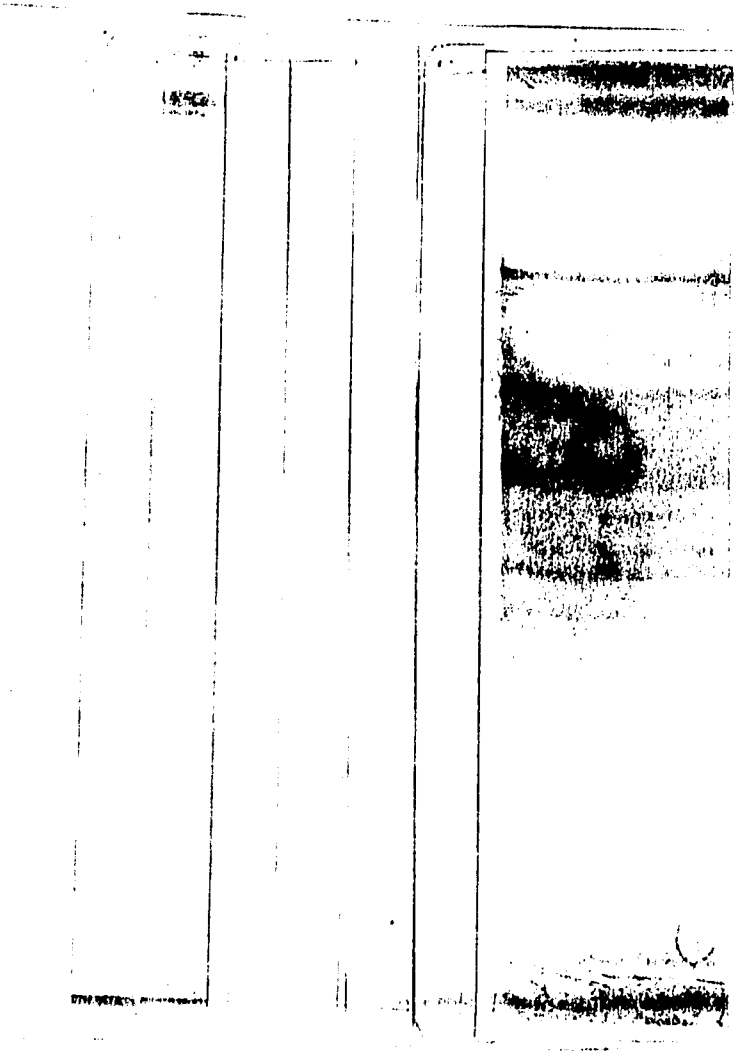
Molecular weight markers (track #1, phosphorylase B, 94,000; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; lactoalbumin, 14,400); Stored delipidized HM2;IMSS (track # 2, reducing conditions; #3, non-reducing conditions); Delipidized HM2:IMSS antigen (track #4, reducing conditions; #5, non-reducing conditions); Freshly prepared delipidized HM2:IMSS antigen (track #6, reducing conditions; #7, non-reducing conditions), and Total HM2:IMSS antigen (track #8, reducing conditions; #9, non-reducing conditions).

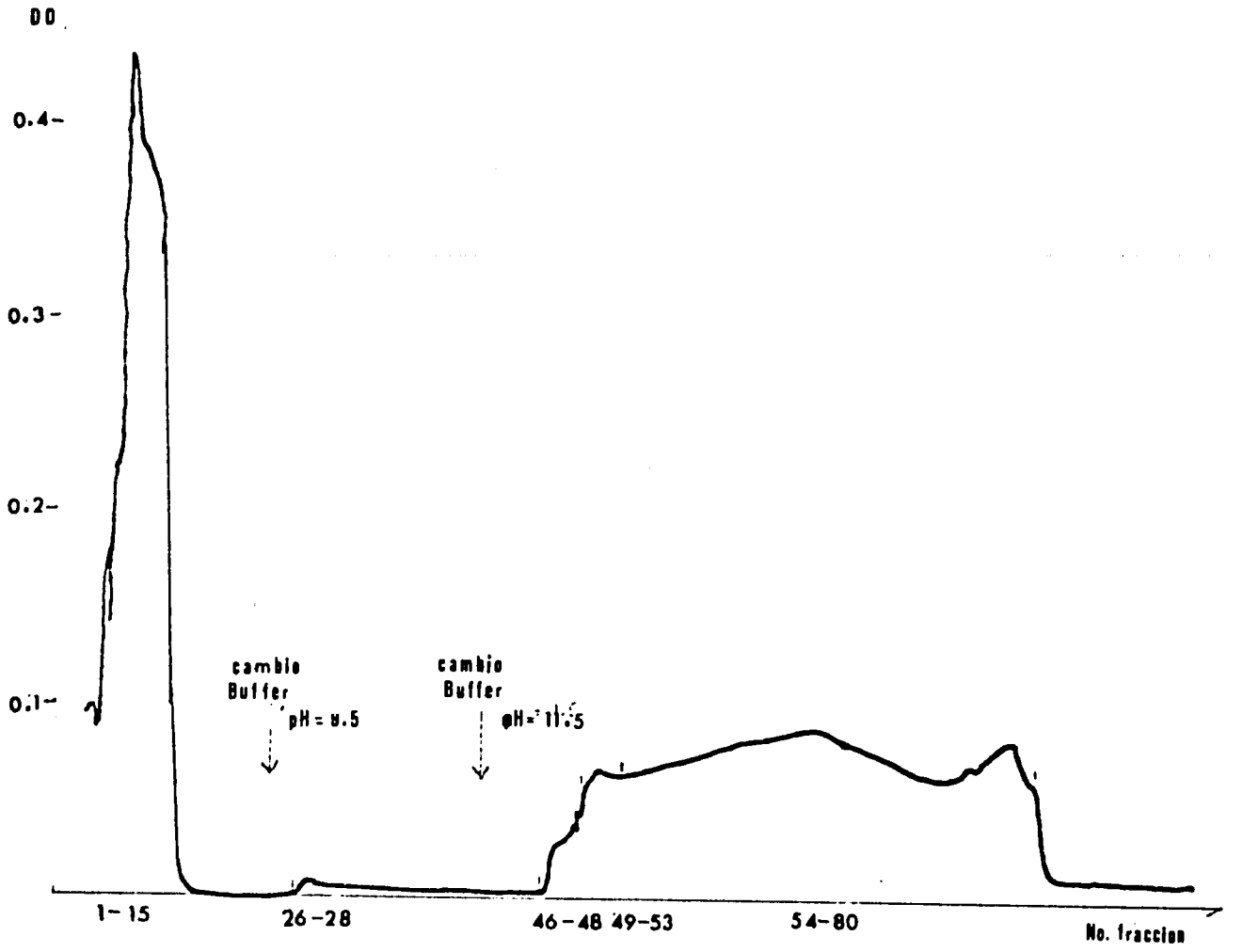
IMMUNOBLOT

E. histolytica HM1:IMSS



E. histolytica HM2:IMSS





Profile of affinity chromatography of total ameba antigen. The material bound to the Sepharose 4B coupled with monoclonal IgM anti-E. histolytica was eluted with 50 mM glycine (pH 9.3) and 50 mM diethylamine (pH 11.5). Arrows indicate change of buffer.

Silver staining of the bands obtained after electrophoresis of the fractions eluted from the Sepharose-monoclonal IgM anti-E. histolytica. Non-specific fraction eluted with 50 mM glycine, pH 9.3 (tracks # 1 and 5); Specific fraction eluted with 50 mM diethylamine, pH 11.5 (tracks #2,3,6,7 and 8); Total E. histolytica HM1:IMSS antigen (track #4); Molecular weight markers (track #9).

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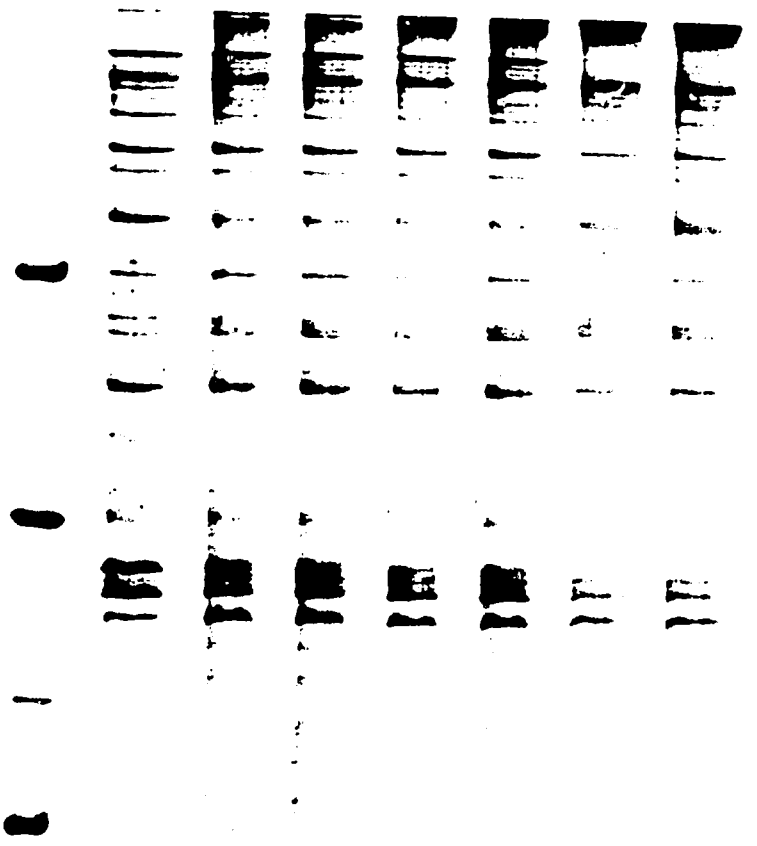

Electrophoresis of E. histolytica membrane antigen in SDS-polyacrylamide gel. Molecular weight markers (track #1, phosphorylase B, 94,000; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; lactoalbumin, 14,400); Non-treated membrane antigen (track #2); Membrane antigen treated with 0.1% (tracks #3 and 4), 0.5% (tracks #5 and 6), or 1.0% (tracks #7 and 8) CHAPS detergent.

EXPERIMENTAL PARASITOLOGY 61, 390–397 (1986)

Entamoeba histolytica: Specific Antigen Recognized by a Monoclonal Antibody

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ORTIZ-ORTIZ, L., XIMÉNEZ, C., MENDOZA, F., MICHALAK, C., MELENDRO, E. I., AND OLIVA, A. 1986. *Entamoeba histolytica:* Specific antigen recognized by a monoclonal antibody. *Experimental Parasitology* 61, 390–397. Specific antigenic determinants on the membrane surface of *Entamoeba histolytica* that distinguish it from other *Entamoeba* species were demonstrated. Evidence for these antigenic determinants was obtained with a monoclonal antibody to *E. histolytica* which showed not only specificity but also sensitivity as demonstrated in enzyme linked immunosorbent assay. Immunofluorescence microscopy showed that the monoclonal antibody recognized an epitope present on the membrane surface of *E. histolytica* trophozoites. The epitope detected by the monoclonal antibody was present in three components of different molecular weight. These components may have a common precursor or may be the result of enzymatic degradation under the conditions tested. © 1986 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Entamoeba histolytica; Entamoeba moshkouski; Entamoeba invadens;* Protozoa, parasitic; Monoclonal antibody; Phosphate buffered saline (PBS); Tween 20 (Tw); Bovine serum albumin (BSA); Enzyme linked immunosorbent assay (ELISA); Concanavalin A (Con A); Sodium dodecyl sulfate (SDS); Polyacrylamide gel electrophoresis (PAGE); Enzyme linked immunoelectrotransfer blot (EITB).

INTRODUCTION

Infections caused by *Entamoeba histolytica* represent a worldwide public health problem, particularly in developing countries, since *E. histolytica* is not limited to tropical areas but is distributed throughout the world (Ortiz-Ortiz and Avella 1984). For the past several years, research on different aspects of amebiasis has increased considerably. The ability to maintain different species and strains of *E. histolytica* in axenic or monoxenic culture has greatly facilitated the study of important physiological aspects of this intestinal parasite and, in turn, of its interrelation with both natural and experimental hosts (Martínez-Palomo 1982). Study of the participation of the host's immune response in resistance to and rejection of amebic infection requires knowledge of the antigen(s) which elicits and immune response. It is assumed that such antigen(s) is, in part, on the surface of the protozoa, since superficial molecules would be the first in contact with the host's immune system and, therefore, those that induce immunity to amebiasis.

E. histolytica has been shown to possess multiple antigenic determinants on its surface; however, these have not been fully characterized (Parkhouse *et al.* 1978; Aley *et al.* 1980). Studies toward the characterization of amebic antigen have been done by using the sera from immune or infected individuals (Trissl 1982). In these investigations, the structural complexity of *E. histolytica* trophozoites was pointed out but antigens specific to the ameba were not characterized.

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The unique homogeneity of monoclonal antibodies (Köhler and Milstein 1975) has made them attractive as a tool, not only to characterize the antigenic composition of ameba but also to purify specific *E. histolytica* antigens. Such antibodies have already been obtained in our laboratory and a clone specific for *E. histolytica* has been partially characterized (López *et al.* 1982).

In this report, we present evidence of the reactivity of a monoclonal antibody to *E. histolytica* with a specific epitope present only on the membrane of this species.

MATERIALS AND METHODS

All strains of the genus *Entamoeba* used in this work were obtained from the culture collection of the Laboratorio de Amibas, Hospital General del Centro Médico Nacional, IMSS, Mexico City, through the generosity of Margarita de la Torre and were maintained axenically in TY1-S-33 medium (Diamond 1968). The strains of *E. invadens*, *E. moshkovskii*, *E. histolytica* related Laredo, and HK9:NIH were originally donated by Dr. Louis S. Diamond, whereas strains HM1:IMSS, HM3:IMSS, and HM38:IMSS were originally isolated in Mexico from patients with amebic dysentery by de la Torre.

The soluble antigen was obtained from trophozoites that had been washed four times with 0.15 *M* sodium chloride solution and then suspended in the same solution. The trophozoite suspension was homogenized by 80 strokes in a glass homogenizer before being centrifuged at 12,500*g* for 20 min. The supernatant of this centrifugation constituted the soluble ameba antigen. Its protein content was standardized (1.0 mg/ml) by determining the protein concentration by the method of Lowry *et al.* (1951).

Plasma membrane antigens were isolated from 10^8 amebae as described by Aley *et al.* (1980). In brief, 10^8 cells were harvested, washed three times with a solution containing 19 m*M* potassium phosphate buffer, pH 7.2, that contained 0.27 *M* NaCl, and centrifuged. The pellet was resuspended in the same buffer but containing 10 m*M* $MgCl_2$ to give 2×10^7 cells/ml and was rapidly mixed with an equal volume of Con A (1 mg/ml) in the same buffer. After 5 min, cells were spun at 50*g* for 1 min to remove excess Con A. The cell pellet was resuspended in 12 ml of 10 m*M* Tris–HCl buffer, pH 7.5, that contained 2 m*M* phenylmethyl sulfonyl fluoride (Tris buffer) and 1 m*M* $MgCl_2$. After swelling for 10 min in the hypotonic buffer, cells were homogenized by 18 to 20 strokes in a glass homogenizer. The homogenate was layered over a two-step sucrose gradient and spun at 250*g* for 30 min. The upper layer consisted of 8 ml of 0.5 *M* mannitol and the lower of 4 ml of 0.58 *M* sucrose, both in Tris buffer. Large plasma membrane fragments and other heavy debris formed a tight pellet at the bottom of the gradient. This pellet was resuspended in 1 ml Tris buffer that contained 1 *M* α-methyl mannoside and was placed on ice for 40 min with occasional mixing.

The plasma membranes, now essentially free of Con A, were diluted with 3 vol of Tris buffer and homogenized by 80 strokes in a glass homogenizer. This second homogenate was layered on a cushion that consisted of 20% sucrose in Tris buffer and was spun for 30 min at 250*g*. Vesiculated plasma membranes floating on top of the sucrose were collected and were concentrated by centrifugation at 40,000*g* for 1 hr. The pellet containing the enriched plasma membranes was resuspended directly in Tris buffer. All samples were frozen at −20 C until used.

Immunization of mice was carried out by giving one dose of 2×10^6 viable *E. histolytica* trophozoites and, 1 month later, administering 100 μg of membranes from *E. histolytica* (Aley *et al.* 1980), both doses given intraperitoneally. Mice were bled at the time of sacrifice and immunoglobulins were obtained by ammonium sulfate precipitation (Campbell *et al.* 1963).

The wells of microtiter plates were coated with 50 μl of 0.01 *M* carbonate buffer, pH 9.6, which contained 10^3 *E. histolytica* trophozoites or 20 μg of either soluble or membrane antigen. The plates were dried overnight at room temperature under vacuum. At the time of use, the plates were rehydrated during 3 min with 0.02 *M* PBS (pH 7.2) containing 0.5% Tw and 0.5% BSA and washed twice with PBS-Tw. Afterward, the wells were filled with a 3% solution of BSA in PBS and were incubated overnight at 4 C to coat the remainder of the active surface, thereby reducing nonspecific protein adsorption. Plates were washed twice with PBS-Tw before use (Saunders 1979).

To determine antibodies to *E. histolytica* by ELISA, plates coated previously with either trophozoites or with soluble or membrane antigen were filled with 50 μl of the test sample, immunoglobulin of mice immunized with *E. histolytica*, to test for the presence of antibodies to *E. histolytica*. The microplates were incubated for 60 min at room temperature and were then washed three times with PBS-Tw-BSA and twice with PBS-Tw. Anti-mouse immunoglobulin coupled to peroxidase (Cappel, Malvern, PA, USA) was then added to each well and incubated at room temperature for 60 min. The plate was washed as before and 50 μl of the substrate, which consisted of 10 ml of 0.1 *M* citrate buffer, pH 4.5, containing 10 mg of *o*-phenylendiamine (Sigma, St. Louis, MO, USA) and 4 μl of a 30% solution of H_2O_2 (Avrameas and Ternynck 1971), was added. All steps were carried out with mild agitation.

Preliminary characterization of monoclonal antibodies specific for *E. histolytica* which were produced

in our laboratory has been reported (López *et al.* 1982). These antibodies were obtained by fusion of spleen cells from *E. histolytica* immunized mice with mouse myeloma X63-Ag8 cells by means of polyethylene glycol (Kennett 1980). Cloned hybridoma lines were obtained from some of the positive wells by limiting dilution on BALB/c thymus cell feeder layers. One of the cloned lines, MC004, was used in all the experiments described in this study. Sufficient quantities of the monoclonal antibody MC004 were obtained by inoculating BALB/c mice intraperitoneally with 0.5 ml of Pristane (Sigma) 5 days prior to their inoculation with 10^6 viable hybridoma cells which secrete monoclonal antibody specific for *E. histolytica*. One to two weeks later, these mice produced abundant ascites fluid, rich in the monoclonal antibody, which was harvested from the peritoneal cavity.

Monoclonal antibodies were purified from the ascites fluid by precipitation with a saturated ammonium sulfate solution (50% final concentration), were resuspended in 0.15 *M* NaCl, and were then dialyzed at 4 C for 1 to 2 days against 0.16 *M* isotonic borate buffer, pH 8.0. The protein concentration was determined by optical density at 280 nm. The monoclonal antibody was of the IgG_1 subclass and contained κ light chains as determined by ELISA assay in which the microplates were coated previously with 20 μg of different specific antisera, namely antibodies to IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, κ, or λ. The antibody to be tested was then added in amounts ranging from 1 ng to 100 μg to microplates coated previously with the preparations of the different *Entamoeba* spp. strains as described above.

Since the monoclonal antibody to *E. histolytica* obtained was of a subclass that binds Protein A, this antibody was purified by affinity chromatography on a Sepharose 4B–Protein A column as described elsewhere (Smith *et al.* 1983). The purified monoclonal antibody to *E. histolytica* was then used in ELISA assays to determine its sensitivity.

For initial characterization of the immunoglobulin chains produced by the monoclonal hybrid, the immunoglobulin was biosynthetically labeled with [^{35}S]methionine. For this purpose, a sterile suspension of 5×10^5 log phase hybridoma cells per ml were washed once in Dulbecco's medium containing 30 mg/ml glutamine and 5% fetal calf serum to which 100 μCi of [^{35}S]methionine were added; cells were incubated at 37 C for 24 hr in a 5% CO_2–air incubator. Finally, 20 min before harvesting the supernatants, 0.1 ml of 10 m*M* methionine was added. The culture supernatants were collected and stored at −20 C. Incorporation of radioactive label into the protein, precipitated by the addition of 1 ml of 20% trichloroacetic acid to a 5 μl aliquot of the supernatant in 1 ml of distilled water, was determined. The mixture was incubated on ice for 10 min, filtered, and a small amount of 95% ethanol was added to facilitate drying. The dried filter was placed in 5 ml scintillation fluid, mixed well by vortexing, and counted in a beta scintillation counter. An aliquot (50 μl) of the culture supernatant was diluted to 100 μl with 50 m*M* Tris–HCl buffer, pH 6.8, containing 5% glycerol, 2% SDS, and 100 m*M* dithiothreitol. Samples were boiled for 5 min and then subjected to electrophoresis on a 10% polyacrylamide gel, under reducing conditions, for 1.5 hr at 40 mA/gel. Some gels were stained with Coomassie blue and destained with 10% acetic acid. After electrophoresis, gels were desiccated, exposed to X-ray films, and stored at −70 C until ready for developing (Haas and Kennet 1980).

Immunofluorescent studies were done using the monoclonal antibody to determine its reactive site on trophozoites of *E. histolytica*. Trophozoites were washed three times with 0.01 *M* PBS containing 0.01% $CaCl_2$ and 0.01% $MgCl_2$, pH 7.2, and were adjusted to 1.5×10^5/0.1 ml. The amebic suspension was centrifuged for 5 min at 200*g* at 4 C. The packed trophozoites were gently resuspended in 150 μl of the monoclonal antibody to *E. histolytica* or the appropriate control medium and were incubated for 15 min at 4 C. Afterward, the amebae were resuspended in 100 μl of a rabbit anti-mouse globulin labeled with fluorescein isothiocyanate (Miles, Naperville, IL, USA) and incubated for 15 min at 4 C. Finally, the suspension was centrifuged at 200*g* for 5 min at 4 C, and the packed trophozoites were suspended in 0.01 *M* PBS containing 4% formaldehyde. Preparations of the amebae were made for observation by fluorescence and phase contrast microscopy.

EITB was used to study antigen antibody pairs. For this purpose, amebic antigen (100 μg), either soluble or membrane portion, was submitted to SDS–PAGE under nonreducing conditions and then transferred onto nitrocellulose sheets by means of a transphor chamber (Hoefer Scientific Instruments, San Francisco, CA, USA) according to the method described previously (Towbin *et al.* 1979). The nitrocellulose sheets were blocked by incubating them (60 min at 37 C) in 0.02 *M* PBS, pH 7.2, containing 3% BSA, 1% gelatin, and 1% sodium azide. The blocked nitrocellulose sheets were then incubated for 90 min at room temperature with either purified monoclonal antibody to *E. histolytica*, normal mouse IgG, or IgG obtained from mice immunized with *E. histolytica*. Thereafter, the sheets were washed two times with PBS-Tw and three times with PBS for 10 min each. The washed nitrocellulose papers were then incubated with a solution of protein A-peroxidase conjugate in 2% PBS-BSA (2.5 μg/ml) for 60 min at room temperature, washed as before, and then exposed for 5 to 10 min to the substrate solution which consisted of 25 mg of 3,3′ diaminobenzidine (3,3′-4,4′-tetra-aminobiphenyl tetra-hydrochloride, 97–99%, Sigma) and 50 μl

of 30% H_2O_2 that had been diluted to 50 ml with PBS, pH 7.2. The nitrocellulose sheets were then thoroughly rinsed with water.

RESULTS

The monoclonal antibody to *Entamoeba histolytica* was tested in an ELISA assay, for specificity against several strains of the genus *Entamoeba*. The monoclonal under study reacted only with the *E. histolytica* strains and had no reactivity with any of the other *Entamoeba* species tested (Table I). Thus, as determined by the ELISA test, the monoclonal antibody to *E. histolytica* was shown to be specific for *E. histolytica* strains. In contrast, mouse polyclonal antibodies against *E. histolytica* reacted with all the strains of the genus *Entamoeba* here tested.

With regard to sensitivity, monoclonal antibody to *E. histolytica* (250 ng) was able to react with as few as one trophozoite per cell and as little as 1.0 ng of either soluble or membrane amebic antigen, as determined by the ELISA test (Table II). The sensitivity showed by the specific monoclonal was higher than that exhibited by the polyclonal *E. histolytica* antiglobulin. The latter globulin was able to detect 10 ng of the different amebic antigens tested.

Radioautography of the slab gel, run under reducing conditions, of the supernatant from the monoclonal antibody producing culture, which had been biosynthetically labeled with [^{35}S]methionine, showed three bands, one corresponding to a protein of 50,000 Da and two to proteins of approximately 25,000 Da (Fig. 1). Parallel gels and radioautographs were run on the labeled supernatant from a culture of the Ig producing myeloma cell line X63-Ag8 and on that from a culture of normal spleen cells. The relative molecular weights of the bands from the MC004 supernatant coincided with those from the X63-Ag8 supernatant. The normal spleen cell culture showed only a slight background band at 50,000 Da.

From the immunofluorescence studies, it was determined that the monoclonal antibody reacted with antigenic determinants located on the surface membrane of the *E. histolytica* trophozoites (Fig. 2). The monoclonal antibody to *E. histolytica* did not react with trophozoites of other species, i.e., the *E. histolytica* related

TABLE I
Specificity of the Monoclonal Antibody to *Entamoeba histolytica*[a]

Globulin tested[b]	Strains of the genus *Entamoeba*						
	E. histolytica						*E. histolytica* related
	HM1:IMSS	HM3:IMSS	HM38:IMSS	HK9:NIH	*E. moshkovskii*	*E. invadens*	Laredo
Monoclonal to *E. histolytica*	+ + +[c] (1.920)[d]	+ + + (ND)[e]	+ + + (ND)	+ + + (ND)	− (0.125)	− (0.102)	− (0.108)
Polyclonal mouse to *E. histolytica*	+ + (1.200)	+ + (ND)	+ + (ND)	+ + (ND)	+ + (0.712)	+ + (0.683)	+ + (0.710)
Normal mouse	− (0.174)	− (ND)	− (ND)	− (ND)	− (0.113)	− (0.099)	− (0.088)

[a] The antibody specificity was measured by ELISA as described under Materials and Methods.

[b] The monoclonal antibody to *E. histolytica*, the polyclonal antibodies, and the normal globulin were used at a concentration of 250 ng/50 μl).

[c] The + and − signs indicate readings made by visual observation.

[d] The number in parentheses indicated readings made in a Behring ELISA Processor M, P21 program (450 nm).

[e] ND = not done.

TABLE II
Sensitivity of the Monoclonal Antibody to *Entamoeba histolytica*[a]

Globulin tested[b]	Ameba antigen tested								
	Trophozoites (No. per well)			Soluble (μg)			Membranes (μg)		
	100	10	1	100	10	1	100	10	1
Monoclonal to *E. histolytica*	1.730[c]	0.750	0.356	1.400	0.850	0.370	1.960	1.530	0.670
Polyclonal mouse to *E. histolytica*	0.920	0.447	0.040	1.020	0.760	0.050	1.190	0.875	0.019
Normal mouse	0.100	0.080	0.050	0.101	0.092	0.045	0.099	0.078	0.060

[a] The antibody sensitivity was measured by the ELISA test as described under Materials and Methods.
[b] The monoclonal antibody to *E. histolytica*, the polyclonal antibodies, and the normal globulin were used at a concentration of 250 ng/50 μl.
[c] Readings were made in Behring ELISA Processor M. P21 program (450 nm).

Laredo strain, *E. moshkovskii*, and *E. invadens*.

The membrane preparation was demonstrated to be a complex mixture by the numerous bands obtained in SDS–PAGE (Fig. 3b). Similar bands were seen when the soluble antigen preparation was used (data not shown). In EITB, the murine polyclonal antibody reacted with many of the proteins in the membrane preparation (Fig. 3c); whereas the monoclonal antibody was found to react with only three of these (Fig. 3d). The normal mouse globulin used as a control showed no reaction (Fig. 3e).

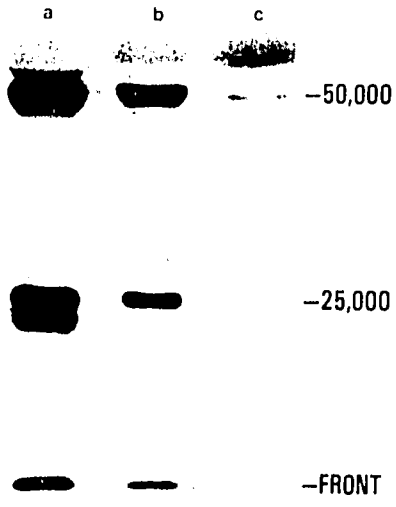

FIG. 1. Radioautography of the SDS–PAGE, run under reducing conditions, of the labeled supernatant obtained from the hybrid culture synthesizing antibody to *Entamoeba histolytica*. (a) Supernatant of the hybrid showing three bands, one corresponding to a protein of 50,000 Da and two, to proteins of approximately 25,000 Da; (b) labeled supernatant from the culture of the Ig producing myeloma cell line X63-Ag8; and (c) labeled supernatant from a normal spleen cell culture.

DISCUSSION

A cell clone actively synthesizing a monoclonal antibody specific for *Entamoeba histolytica* trophozoites was obtained. By radioautography of the SDS–PAGE of labeled culture supernatant, it was shown that the hybrid synthesized and secreted antibody. The bands were located in positions corresponding to those for one heavy and two light immunoglobulin chains. The presence of two light chains is explained by the fact that the globulin produced by the myeloma cell line used in this fusion is secreted in addition to the monoclonal antibody (Köhler *et al.* 1976).

In ELISA, the polyclonal antibody reacted with several strains of the genus *Entamoeba* which points out not only the lack of specificity for *E. histolytica* but also the sharing of antigenic determinants

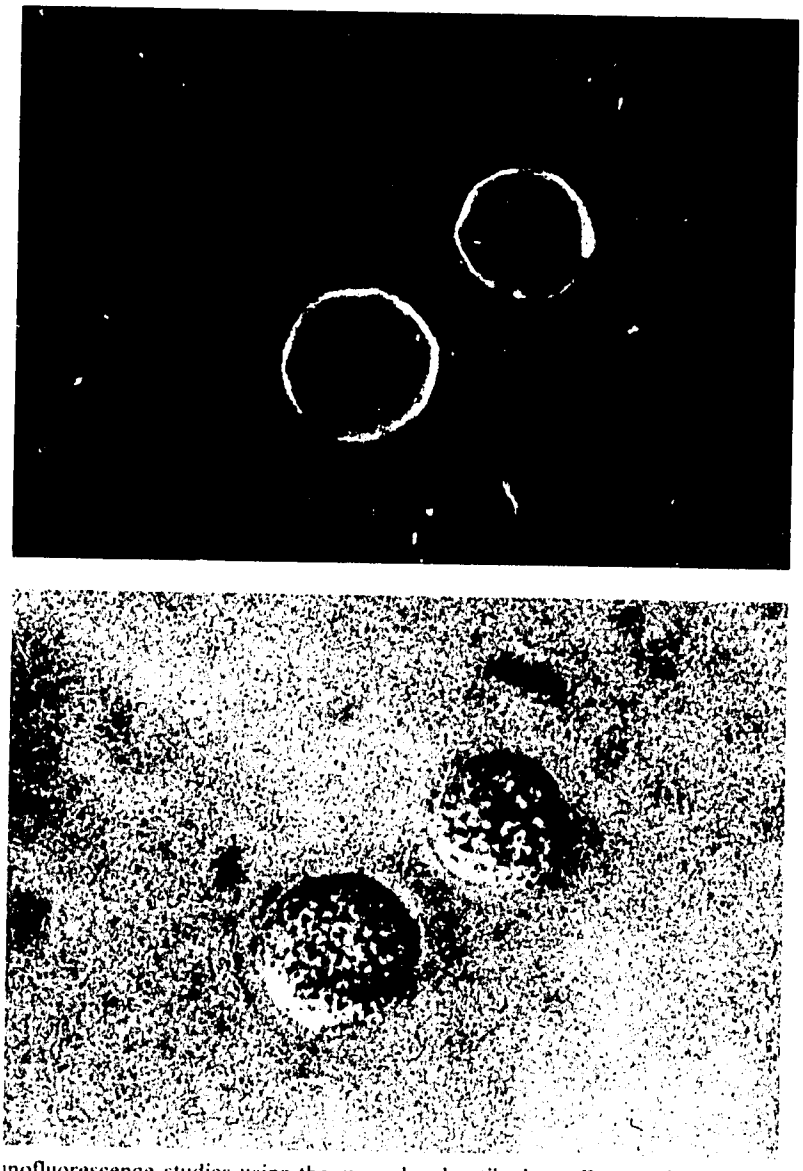

FIG. 2. Immunofluorescence studies using the monoclonal antibody to *Entamoeba histolytica*. The monoclonal reacted with antigenic determinants located on the surface membrane of the *E. histolytica* trophozoites (upper frame), and the same trophozoite seen under the phase-contrast microscope (lower frame).

among strains. In marked contrast, the monoclonal antibody did not react with the other *Entamoeba* species tested, *E. histolytica* related Laredo, *E. moshkovskii*, and *E. invadens* species. The monoclonal antibody to *E. histolytica* also showed sensitivity since it was able to detect only one trophozoite or only 1.0 ng of either soluble or membrane amebic antigen. By immunofluorescence, the monoclonal antibody to *E. histolytica* appeared to be specific for antigen associated with the plasma membrane of *E. histolytica* since it did not react with any of the other *Entamoeba* species assayed.

Comparison of the reactivities in EITB

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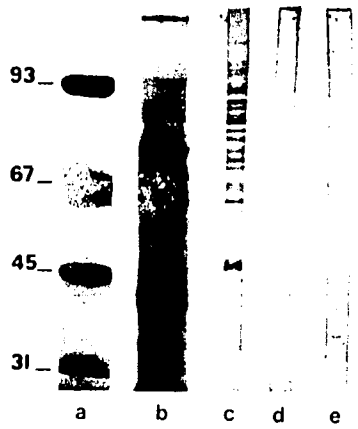

FIG. 3. Electrophoretic analyses of the antigen obtained from the membrane of *Entamoeba histolytica*. SDS–PAGE analyses of (a) molecular weight markers phosphorylase B (93,000), bovine serum albumin (67,000), ovalbumin (45,000), and carbonic anhydrase (31,000); and (b) the membrane antigen from *E. histolytica*. EITB analyses of the membrane antigen developed with (c) the mouse polyclonal antiglobulin to *E. histolytica*, (d) the mouse monoclonal antiglobulin to *E. histolytica*, and (e) with normal mouse globulin. Lanes (a) and (b) were stained with Coomassie blue.

of the polyclonal and monoclonal antibodies with the membrane preparation points out the specificity of the monoclonal antibody to *E. histolytica*. The monoclonal antibody recognized an antigenic determinant present in only three of the many polypeptides in the plasma membrane preparation. These three polypeptides of distinct molecular size may have a precursor–product relationship a finding similar to that described for other protozoa (Holder and Freeman 1982). A more likely possibility may be the enzymatic degradation of the amebic antigen that could result in the conversion of a single polypeptide into three independent polypeptides as detected in our EITB experiments; such degradation was seen with the proteolysis of human serum albumin (Reichlin 1975).

The presence of bands in the soluble antigen preparation similar to those recognized by the monoclonal antibody in the surface membrane fraction may be explained by partial solubilization of surface membrane components during preparation of the soluble antigen. This point is further supported by the positive results obtained in ELISA as mentioned above.

If it is possible to gain access to cysts from both *E. histolytica* and *E. coli* strains, we plan to determine if this antibody can distinguish between *E. histolytica* and *E. coli* cysts. We also intend to obtain antigen to be tested with specific monoclonal antibody to *E. histolytica* by freeze-thawing the cysts.

In summary, the findings here reported demonstrate for the first time specific antigenic determinants on the membrane surface of *E. histolytica* that enable us to distinguish it from other *Entamoeba* species tested. The availability of specific monoclonal antibody to *E. histolytica* surface antigens has many applications, such as the immunohistologic localization of trophozoites in tissue sections, detection of antigens in stool samples, in amebic liver abscess aspirates, or in the circulation during antigenemia in amebiasis.

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Caracterización inmunológica de un anticuerpo monoclonal específico para *E. histolytica*[1]

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Resumen: En este reporte informamos sobre la caracterización de un anticuerpo monoclonal contra *E. histolytica*. El anticuerpo mostró ser específico para *E. histolytica* ya que no reaccionó por inmunofluorescencia ni por la prueba de ELISA como otras *Entamoeba*, específicamente con *E. moshkovskii*, *E. invadens* y de la cepa Laredo de tipo *E. histolytica*. Los estudios de inmunoelectrotransferencia de un antígeno de membrana de *E. histolytica* indican que el anticuerpo monoclonal reconoce tres polipéptidos de diferente peso molecular, que pueden ser el resultado de un precursor común o debido a un artefacto causado por la degradación enzimática de uno de los componentes de la amiba.

Las infecciones ocasionadas por *Entamoeba histolytica*, representan un problema de salud pública en diversas partes del mundo, ya que este parásito tiene una distribución cosmopolita.[1] En los últimos años, el interés por realizar investigaciones en los diferentes aspectos de la amiba, ha aumentado considerablemente. La posibilidad de mantener a este protozoario, así como a diferentes especies del mismo, en cultivos axénicos o monoxénicos, ha facilitado el estudio tanto de la biología del mismo como de la relación huésped parásito, característica de este tipo de infecciones.[2]

El estudio de la participación de la respuesta inmune del huésped en la resistencia y eliminación de la infección requiere del conocimiento del o los antígenos que inducen una respuesta inmune. Se piensa que tales antígenos se localizan fundamentalmente sobre la superficie del microorganismo, ya que son estas moléculas las primeras en ponerse en contacto con el sistema inmune del huésped, e inducir una respuesta inmune que puede ser efectiva en la eliminación del parásito.

Entamoeba histolytica posee múltiples de-

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terminantes antigénicos sobre su superficie; sin embargo, éstos no han sido identificados en su totalidad.[3] Los estudios de caracterización de los antígenos amibianos se han hecho utilizando antisueros de individuos infectados o de animales inmunizados.[4] Estos estudios han puesto en evidencia la complejidad estructural de *E. histolytica.*

Uno de los avances más importantes en la última década ha sido, sin duda, la obtención de anticuerpos monoclonales por Kohler y Milstein,[5] como resultado de la fusión de una célula mielomatosa o con otra célula linfoide de un animal inmune. La disponibilidad de esta tecnología ha permitido, entre otras cosas, el desarrollo de pruebas diagnósticas en el caso de enfermedades infecciosas así como el estudio de los antígenos involucrados en la patogenicidad de muchos parásitos que presentan estructuras antigénicas muy complejas.

Ya que *E. histolytica* posee múltiples antígenos, algunos de los cuales comparte con otras especies de *Entamoeba,* nos propusimos emplear la tecnología de hibridomas para la producción de un anticuerpo monoclonal específico de especie que nos permitiera diferenciar de manera segura entre la especie *histolytica* y otras especies de *Entamoeba* no patógenas. Este sería un primer paso para poder establecer las condiciones necesarias en la elaboración d una prueba diagnóstica confiable. En este informe se presentan los resultados de la caracterización inmunológica de un anticuerpo monoclonal específico para *E. histolytica.*

MATERIAL Y METODOS

TROFOZOITOS

Se utilizaron trofozoítos de: *E. histolytica,* cepas HM1: IMSS, HM3: IMSS, HM38: IMSS, HK9: NIH; *E. invadens, E. moshkovskii* y la cepa Laredo, *Entamoeba* clasificada como semejante a la especie *histolytica.* Todas las cepas y especies se mantuvieron en cultivos axénicos en el medio de Diamond TY-I[2] y se colectaron en la fase de crecimiento exponencial.

ANTIGENO DE MEMBRANAS CITOPLASMICAS DE *E. histolytica*

La obtención de membranas citoplásmicas de *E. histolytica* se realizó por medio del método descrito por Aley y colaboradores.[6] Para ello se lavaron 10^8 trofozoítos por centrifugación a 200 g durante 10 minutos a 4 °C con una solución amortiguadora de fosfato de potasio 19 mM, pH 7.2 adicionada de 0.27 M de NaCl. El paquete se resuspendió en la misma solución adicionada con 10 mM de $MgCl_2$ para dar una suspensión celular de 2×10^7 células/ml, a continuación se añadió un volumen igual de una solución de 1 mg/ml de Concanavalina A (Con-A). Después de 5 min de incubación en baño de hielo las células aglutinadas se centrifugaron a 50 g durante 1 min para quitar el exceso de Con-A. El paquete celular se resuspendió en 12 ml de una solución amortiguadora de Tris-HCl[10] mM, pH 7.5 a la que se añadió inhibidores de proteasas. Después de 10 min las células se hemogeneizaron manualmente, este homogenado se colocó sobre un gradiente de manitol 0.5 M y sacarosa 0.58 M ambos en solución de Tris-HCl. Los fragmentos grandes de membranas y otros restos celulares formaron un paquete en el fondo del grandiente, el cual se resuspendió en 1 ml de solución de Tris con α-metil manósido 1 M y se incubó a 4 °C durante 40 min. Las membranas plasmáticas libres de Con-A se diluyeron en tres volúmenes de Tris y se homogeneizaron.

Este segundo homogenado se pasó por un colchón de sacarosa a 20% centrifugando a 250 g durante 1 h. Las membranas plasmáticas se colectaron y ultracentrifugaron a 40,000 g durante 1 h. El paquete se resuspendió en solución de Tris y se congeló a −20 °C hasta el momento de su uso.

PREPARACION DE PLACAS DE MICROTITULACION CON ANTIGENOS O TROFOZOITOS DE *Entamoeba* PARA DETERMINACION DE ANTICUERPOS

Las placas de microtitulación se cubrieron con 20 μg de antígeno o 10^3 trofozoítos suspendidos en 50 μl de una solución amortiguadora de carbonatos 0.01 M pH 9.6 y se secaron al vacío en un desecador durante toda la noche. En el momento de ser utilizadas, las placas se hidrataron con una solución amortiguadora de fosfatos (PBS) 0.02 M pH 7.2 conteniendo 0.5% de Tween 20 y 0.5% de seroalbúmina bovina (PBS-Tw-BSA), y se lavaron dos veces con PBS-Tw. Con el objeto de bloquear los

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sitios activos remanentes los pozos se llenaron con una solución a 3% de BSA en PBS y se incubaron a 4 °C durante toda la noche; posteriormente se lavaron dos veces con PBS-Tw antes de adicionar el primer anticuerpo.[7]

DETERMINACION DE ANTICUERPOS ANTI-*E. histolytica* POR MEDIO DE METODO INMUNOENZIMATICO (ELISA)

Para tal objeto se usaron las placas de microtitulación previamente cubiertas con el antígeno o los trofozoítos, a las cuales se les adicionaron 50 μl por pozo de las diluciones de los sueros en estudio o de la IgG purificada del anticuerpo monoclonal. Las placas se incubaron a temperatura ambiente durante 1 h, en agitación, después de lo cual se lavaron como se indicó anteriormente. A continuación se adicionaron 50μl por pozo de una solución de antiinmunoglobulina de ratón marcada con la enzima peroxidasa. Se incubó nuevamente durante 1 h y se procedió a lavar las placas como en el paso anterior para finalmente añadir 50 μl del sustrato, que consistió en 10 ml de una solución amortiguadora de citratos 0.1 M pH 4.5 adicionada de 10 mg de σ-fenilendiamina (SIGMA) y 4 μl de H_2O_2 al 30%. La reacción se dejó transcurrir durante 3 min y se bloqueó con 200 μl de una solución 1 M de H_2SO_4 por pozo.[8]

PURIFICACION DE ANTICUERPOS MONOCLONALES

Para obtener suficientes cantidades de anticuerpos monoclonales, las clonas productoras de anticuerpos se expandieron y se inocularon en la cavidad peritoneal de ratones BALB/c tratados con Pristan (Aldrich). Aproximadamene 10 días después de la inoculación, los ratones presentaron líquido ascítico en la cavidad abdominal; este líquido se drenó por punción peritoneal. El líquido ascítico se centrifugó a 2,000 g durante 30 min a 4 °C con el objeto de eliminar las células y eritrocitos; el sobrenadante se almacenó en alícuotas a −20 °C. Para purificar los anticuerpos a partir del líquido de ascitis, se precipitó a 4 °C con una solución saturada de sulfato de amonio a una concentración final del 50% v/v. Después de la precipitación las proteínas se dializaron en solución de boratos 0.016 M isotónica pH 8.0. Ya que previamente se determinó la clase y subclase de inmunoglobulina en cuestión, se procedió a purificar las IgG mediante una columna de afinidad de Sefarosa-Proteína A.

ESTUDIOS DE INMUNOFLUORESCENCIA DE TROFOZOITOS DE *E. histolytica* CON ANTICUERPOS MONOCLONALES ESPECIFICOS

Para determinar la reactividad de los monoclonales se realizaron estudios de inmunofluorescencia indirecta. Los trofozoítos de *E. histolytica* se lavaron 3 veces por centrifugación con PBS 0.01 M pH 7.2 la cual contenía 0.01% de $CaCl_2$ y 0.01% de $MgCl_2$, se ajustaron a una concentración de 1.5×10^5 en 100 μl y se centrifugaron a 200 g a 4 °C durante 5 min. El paquete de trofozoítos se resuspendió en 100 μl de la dilución del anticuerpo monoclonal o las diluciones de antisueros utilizadas como controles, y se incubó durante 15 min a 4 °C. Posteriormente se lavó de nuevo para eliminar el anticuerpo libre; se añadieron 100 μl de la antiinmunoglobulina marcada con fluoresceína, se incubó durante 15 min y se lavó por medio de centrifugación como se indicó previamente. El paquete de trofozoítos obtenido en

CUADRO I
ESPECIFICIDAD DE LOS ANTICUERPOS MONOCLONALES (ELISA)

Clona	*Entamoeba histolytica* HM1: IMSS	HM3: IMSS	HM38: IMSS	*E. moshkovskii* HK-9-NIH	CST	*E. invadens*	*E. histolytica* cepa Laredo
MC001	+	+	+	+	+	+	+
MC002	+	+	+	+	+	+	+
MC003	++	++	++	++	+	+	+
MC004	++++	++++	++++	++++	—	—	—
MC005	+++	+++	+++	+++	+++	+++	+++
MC006	+	+	+	+	+	+	+
control positivo	++	++	++	++	++	++	++
control negativo	—	—	—	—	—	—	—

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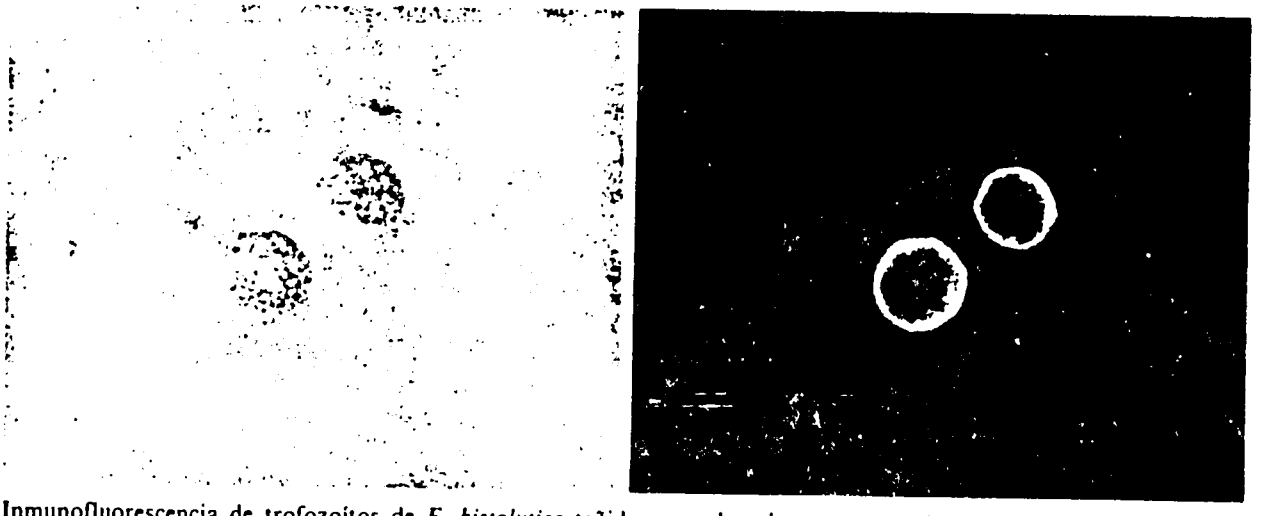
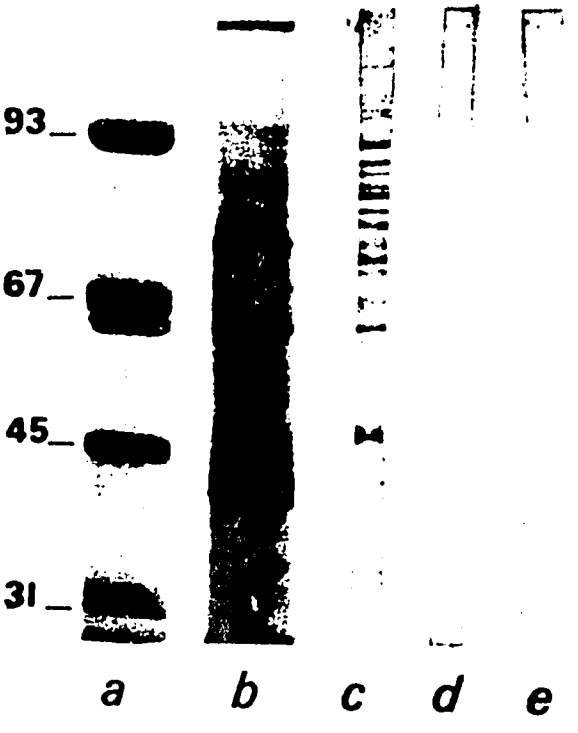
FIGURA 1



Inmunofluorescencia de trofozoítos de *E. histolytica* teñidas con el anticuerpo monoclonal anti-*E. histolytica* y un segundo anticuerpo anti-globulina de ratón acoplada a isotiocianato de fluoresceína: Amibas observadas en microscopio de contraste de fases (izquierda), y el mismo campo en microscopio de fluorescencia (derecha).

FIGURA 2



Inmunoelectrotransferencia de antígeno de membrana de *E. histolytica:* (a) Marcadores de peso molecular; (b) Antígeno teñido con azul de Coomasie; (c) Antígeno revelado con anticuerpo policlonal de ratón BALB/c; (d) Antígeno revelado con anticuerpo monoclonal anti-*E. histolytica* (MC004), y (e) Antígeno revelado con inmunoglobulinas normales de ratón BALB/c.

la última centrifugación se resuspendió en una solución al 4% de formaldehido en PBS 0.01 M, se montaron las preparaciones y se hizo la observación.

INMUNOTRANSFERENCIA DE LOS ANTIGENOS DE *E. histolytica*

Se hizo el análisis del patrón electroforético de los antígenos de membrana citoplásmica *E. histolytica* HM1: IMSS. Los geles de poliacrilamida-SDS se transfirieron a papel de nitrocelulosa siguiendo el método de Towbin y col.[9] con algunas modificaciones. El papel de nitrocelulosa previamente bloqueado se hizo reaccionar con el anticuerpo monoclonal, con la IgG de ratón BALB/c inmunizado con *E. histolytica* o con la IgG de ratón BALB/c normal. Para evidenciar la reacción se utilizó proteína A de estafilococo acoplada a la enzima peróxidasa, y como sustrato la diaminobenzidina (50 mg) y H_2O_2 al 3% y (100 μl) en PBS 0.02 M pH 7.2.

RESULTADOS

ESPECIFICIDAD DE LOS ANTICUERPOS MONOCLONALES ANTI-*E. histolytica*

Los resultados de la reactividad de los anticuerpos monoclonales ensayados contra las diferentes especies y cepas de *Entamoeba*, me-

diante la prueba de ELISA se muestran en el cuadro I. Como se puede observar, el anticuerpo 004 reacciona únicamente con las cepas de *E. histolytica* estudiadas y no muestra reactividad con ninguna de las otras especies de *Entamoeba*, por lo tanto es especie específico.

INMUNOFLUORESCENCIA DE TROFOZOITOS DE *E. histolytica* CON ANTICUERPOS MONOCLONALES ESPECIFICOS

El estudio de inmunofluorescencia indirecta con un cuerpo monoclonal 004 muestra que los determinantes antigénicos que son reconocidos por este anticuerpo se encuentran distribuidos de manera homogénea sobre la superficie de los trofozoítos a diferencia del patrón de fluorescencia que se observa cuando se utilizaron IgG de animales inmunes (figura 1).

INMUNOTRANSFERENCIA DE PROTEINAS DE MEMBRANAS DE *E. histolytica*

La inmunotransferencia de proteínas de membrana de *E. histolytica* HM1: IMSS y su reactividad con el monoclonal específico, muestra que dicho anticuerpo reacciona con tres bandas proteínicas. Este hallazgo es diferente del que presentan las IgG de ratón BALB/c inmune, las cuales reconocen múltiples proteínas de membrana (Figura 2).

DISCUSION

El anticuerpo monoclonal 004 es capaz de diferenciar por el método de ELISA entre la especie *histolytica* y otras especies de *Entamoeba*. Este anticuerpo parece estar dirigido a determinantes antigénicos presentes en la superficie celular, como se demuestra en el estudio de inmunofluorescencia indirecta.

La técnica de inmunotransferencia de proteínas en papel de nitrocelulosa mostró que el anticuerpo monoclonal específico reconoce un epitope presente en tres polipéptidos de diferente peso molecular obtenidos de membranas de *E. histolytica*. Es posible que estos polipéptidos puedan generarse a partir de un precursor común, tal como ha sido descrito en otros parásitos,[10] o muy probablemente que estos péptidos sean productos de degradación enzimática del antígeno amibiano, originando 3 bandas peptídicas independientes; este tipo de degradación ha sido observado en la proteolisis de seroalbúmina humana.[10]

La posibilidad de discriminar entre diferentes especies de *Entamoeba* ofrece ventajas para el diagnóstico de amibiasis, principalmente en la forma intestinal de la enfermedad. Un paso obligado será el de investigar si este anticuerpo puede reaccionar con la forma quística del parásito mediante el rompimiento de dichos quistes y tratar de ver si a este nivel se conserva la especificidad de especie observada con los trofozoítos.

Ximénez C, Mendoza F, Michalak C, Melendro EI, Oliva A, Ortiz Ortiz L: *Inmunologic characterization of an specific monoclonal antibody for* E. histolytica. Salud Pública Méx., 1985; 27: 235-240.

Summary: In this report we inform on the characterization of a monoclonal antibody to *E. histolytica*. The monoclonal antibody is shown to be specific for *E. histolytica* since it does not react with other *Entamoeba* tested, namely, *E. moshkovskii*, *E. invadens*, and the *E. histolytica*-like Laredo strain. The immunoblot of an antigen obtained from *E. histolytica* membranes and developed with the monoclonal antibody shows three bands. The presence of an epitope in three componentes of different molecular weight can be the result of a common precursor or due to enzymatic degradation of an amebic component.

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DIAGNOSIS OF *ENTAMOEBA HISTOLYTICA* IN FECES BY ELISA[1*]

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* U.S. Patent applied for.

ABSTRACT

A diagnostic test for intestinal amebiasis was developed by using an ELISA that incorporated a well-defined monoclonal antibody specific for a membrane antigen of *E. histolytica*. The ELISA was found to be sensitive and quite specific for *E. histolytica*, since there were no cross reactions with other parasites, especially *E. coli*, with which *E. histolytica* is often confused. In addition, this test detected not only *E. histolytica* trophozoites but also cysts. This technique will be of great value in the rapid and accurate diagnosis of *E. histolytica* in human fecal material.

KEY WORDS

Entamoeba histolytica; monoclonal antibody; parasitic diagnosis; ELISA.

INTRODUCTION

Worldwide, infections caused by *Entamoeba histolytica* are an extremely important public health problem. Although this protozoan infects the large intestine, it can invade other organs such as the liver, brain, skin, genitals, lung, etc. (1). Diagnosis of intestinal amebiasis by microscopic identification of cysts or the vegetative form of *E. histolytica* has the disadvantages of requiring not only personnel with experience in such identification but also samples taken on three different days which, in practice, often means a delay in diagnosis due to lack of adequately supplied samples. Many attempts have been made to adapt an immunoenzymatic assay (ELISA) to the detection of *E. histolytica* antigen(s) in fecal material (2-4). In the majority of such attempts, the use of polyclonal antibodies produced relatively unreliable results because of lack of specificity (5,6). In one report, monoclonal antibodies were used; however, the monoclonals were not immunochemically characterized to determine specificity (7).

In this work, we report the development of a diagnostic test for intestinal amebiasis by means of an ELISA in which a well-defined monoclonal antibody, specific for a membrane antigen of *E. histolytica* (8) was used.

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MATERIALS AND METHODS

Trophozoites of E. histolytica. Trophozoites of the pathogenic *E. histolytica* strain HM1:IMSS were maintained in axenic culture in Diamond TY-1 (9) and were harvested for use in the exponential phase of growth.

Isolation of plasma membrane from E. histolytica. Cytoplasmic membranes from *E. histolytica* were obtained by the method of Aley *et al*. (10).

*Production of polyclonal anti-*E. histolytica. New Zealand white rabbits were immunized by injection in the footpadas with 1.0 ml of an emulsion consisting of 5 X 10^6 viable trophozoites in 0.5 ml 0.15 M NaCl and 0.5 ml Freund's incomplete adjuvant (Difco, Detroit, MI). These animals were again injected intramuscularly and subcutaneously with the same quantity of viable trophozoites (without adjuvant) 8 and 15 days later. When these animals demonstrated the production of serous antibodies against membrane antigen as determined by double immune diffusion (11), they were bled. The immunoglobulin fraction was purified from the separated sera by precipitation with ammonium sulfate (12).

*Production of monoclonal anti-*E. histolytica. BALB/c mice were immunized by intraperitoneal (i.p.) injection of 2 X 10^6 viable *E. histolytica* trophozoites. One month later, they were injected i.p. with 100 μg of *E. histolytica* membrane antigen. Four days after this last injection, the animals were killed and the spleens removed. These immune spleen cells were fused with SP2/O myeloma cells (ratio 100:1) by using polyethylene glycol 4, 00 (Sigma). Production of antibodies by hybrid cells was determined by ELISA. Those hybrid cells producing specific anti-

E. histolytica were cloned in soft agar (13). Production of specific immunoglobulin in the resulting colonies was determined by ELISA. Positive clones were expanded in vitro and thereafter in vivo, in ascitis form in BALB/c mice previously inoculated i.p. with Pristan (Sigma). Eight days later, the ascitis liquid was obtained and the immunoglobulin fraction was recovered.

That this monoclonal antibody is specific to strains of E. histolytica has been demonstrated (8). In an ELISA, this monoclonal antibody was shown to react with soluble antigen from E. histolytica strains HM1:IMSS, HM3:IMSS, HM38:IMSS, and HK9:NIH but not from E. moshkovskii, E. invadens, or E. histolytica related Laredo strain. This specificity was also shown in immunofluorescence studies, in which this monoclonal antibody reacted with trophozoites from E. histolytica but not from E. moshkovskii, E. invadens, or E. histolytica related Laredo strain.

Preparation of the anti-E. histolytica-biotin conjugate. The monoclonal antibody was dialyzed against 0.1 M $NaHCO_3$, pH 8.0. Thereafter, the protein concentration was determined (14) and, was adjusted to 100 µg/ml with 0.1 M $NaHCO_3$. For each 100 µg of antibody, 100 µl of DMSO containing 1.1 mg-hydroxysuccinimido-biotin (Sigma) was added. After the mixture had been incubated (2h, 25°C), the reaction was stopped by the addition of 0.1 volumes of 1 M NH_4Cl, pH 7.2. The resulting mixture was dialyzed against 0.01 M phosphate buffer containing 0.15 M NaCl (PBS) pH 7.2 (4°C, 24 h) (15).

Preparation of fecal samples. Frozen fecal samples (272) were received from the Hospital General del Centro Medico del IMSS and from the Instituto Nacional de Pediatria, Mexico City. The samples were coded in a double blind study. After the data from ELISA were obtained, the results were compared to that from microscopic studies of samples prepared by the Faust method (16). For ELISA, each fecal sample was homogenized in PBS, pH 7.4, which contained sodium dodecyl sulfate (0.05%), and three concentrations (50 mg, 5 mg, and 0.5 mg per ml) were tested.

ELISA determinations of E. histolytica in feces. Each well of polystyrene plates (Immulon II, Dynatech Laboratories, Alexandria, VA) was coated by the addition of 10 μg of polyclonal anti-E. histolytica in 100 μl 0.1 M carbonate buffer, pH 9.6 and by drying the plates in a dessicator under vacuum. Previous testing of varied amounts (0.01, 0.1, 1.0, 10, and 100 μg) of this antibody showed 10 μg to be optimal. At the start of the assay, the wells were washed three times by adding PBS, pH 7.4 containing 0.05% Tween 20 (PBS-Tw) (200 μl per well, 3 min; 20°C). The wells were then blocked by the addition of 1% (w/v) bovine serum albumin (BSA) in PBS (200 μl per well; 4h; 20°C; gentle agitation).

The sensibility of the test was determined by parallel experiments in which E. histolytica membrane antigen (0.1 to 100 μg) and E. histolytica trophozoites (1 to 1,000) were used. Therefore, to the appropriate plates, known quantities of either membrane antigen, trophozoites, or feces were added. After incubation for 1 h at 37°C and 2 h at 20°C (with agitation), the plates were washed three times with PBS-Tw containing 0.05% BSA (PBS-Tw-BSA) for 3 min before each change. The monoclonal anti-E. histolytica-biotin conjugate (50 μl) was added to each well and the plates were incubated 1 h at 37°C and 2 h at 20°C, with gentle agitation. The plated were then washed with PBS-Tw-BSA as before. Immediately thereafter, 50 μl of streptavidin-peroxidase (Amersham International, UK) which had been diluted 1:400 in PBS containing 1% BSA was added to each well and the plates were incubated for 1 h at 37°C. The plated were then washed 5 times for 3 min with PBS-Tw and 50 μl of substrate was

added to each well. The substrate was prepared by adding 10 mg o-pheylenediamine and 4 ul 30% H_2O_2 to 10 ml of 0.1 M citrate buffer, pH 4.5. The plates with substrate were incubated for 4 min at 20°C in the dark. To stop the reaction, 200 ul of 1 M H_2SO_4 was added to each well. The plates were then read at 495 nm in an ELISA processor M (Behring, Marlburg, West Germany).

RESULTS

The ELISA in which our monoclonal anti-*E. histolytica* was used was sensitivite, detecting as little as 0.1 ug of *E. histolytica* membrane antigen and ten *E. histolytica* trophozoites. ELISA reading ranged from 0.095 to 0.530 for the amounts (0.1 to 10 ug) of membrane antigen used (Fig. 1A). Although the use of one trophozoite produced a reading close to background values, 10 to 1,0000 trophozoites gave detectable readings (0.057 to 0.307) (Fig. 1B).

This ELISA proved useful for the detection of *E. histolytica* in feces. Of the initial 272 samples studied, only 20 were positive for *E. histolytica* as determined by ELISA. These findings were confirmed by microscopy: of the 20 samples that were positive for *E. histolytica*, two contained trophozoites and 18 contained *E. histolytica* cysts. Also, of these 20, six also contained other parasites (Table I). Fifty other samples were found to be infected with one or more parasites but these were free of *E. histolytica*. No parasites were detected in the remaining 202 samples.

The optical density readings of the three dilutions of the 20 *E. histolytica* posivite fecal samples were compared among themselves and to the readings of the negative controls. Although the

readings of the three dilutions gave values that were significantly different from those of the negative controls (p 0.001), they did not differ among themselves (p 0.05) (Table II and Fig. 2).

Since these preliminary studies indicated that the quantity of stool sample to be analyzed was not critical for evaluating the presence of *E. histolytica*, in a later study the amount of feces was not weighed. In this study, 429 samples were examined and 26 were positive for *E. histolytica* as determined by ELISA. By microscopy, only 13 of these 26 samples and no others were positive (Table III). When the clinical records were examined to determine the reability of the ELISA test, it was found that the symptoms of those patients with the positive stool samples were compatible with a diagnosis of amebic infection. Here again, by microscopy, some samples positive for *E. histolytica* contained other parasites, while other negative samples were found to be infected with one or more parasites but to be free of *E. histolytica*. No parasites were detected in the remaining 213 samples. Therefore, in addition to being sensitive, the ELISA assay is quite specific for *E. histolytica* since there was no cross-reaction with other parasites and there were no false positives or false negatives.

DISCUSSION

The ELISA method used in this work showed that the monoclonal anti-*E. histolytica* could detect the presence of amebic antigen in fecal samples and was sufficiently sensitive to detect 0.1 g of this antigen or ten or more *E. histolytica* trophozoites.

As stated in Materials and Methods, this monoclonal antibody has been shown to have the necessary specificity to distinguish between *E. histolytica* and other *Entamoeba* species (8). This specificity was also demonstrated here by the lack of reactivity with the other parasites, especially *E. coli*, with which *E. histolytica* is often confused. Repetition of assays on samples which had been frozen for over 30 days showed no change in the results.

This technique, therefore, is not only sensitive and highly specific but also rapid and precise. Since it obviates the need of microscopic studies and serial samples, a greater number of samples can be processed in less time. Because this ELISA also detected *E. histolytica* cysts, the identification of asymptomatic carriers is facilitated. This technique will be of great value in the rapid and accurate diagnosis of *E. histolytica* in fecal material.

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Table I

Determination of the Presence of *E. histolytica* in Feces by Microscopy and by ELISA

Parasite	Microscopy	ELISA
Parasites absent	202/272	252/272
Parasites present	70[a]/272	20/272
E. histolytica	20[b]/70	20[a]/70
Other parasites:	50/70	0[c]/70
E. coli	14/70	0/70
G. lamblia	14/70	0/70
E. nana	11/70	0/70
I. butschlii	4/70	0/70
Ch. mesnili	4/70	0/70
A. lumbricoides	3/70	0/70
T. trichura	1/70	0/70

[a] Some samples contained more than one parasite.

[b] Same 20 samples which were positive in ELISA

[c] Negative ELISA implies no cross reaction

Table II

Sensitivity of ELISA to Different Concentrations of *Entamoeba histolytica* Positive Feces

Group	Fecal sample (mg/ml)	No. of samples assayed	Optical Density$_{495\ nm}$ ± S.E.	p[a]
1	50.0	20	0.151 ± 0.024	0.001
2	5.0	20	0.111 ± 0.011	0.001
3	0.5	20	0.086 ± 0.011	0.001
4	Blank[b]	20	0.025 ± 0.005	-
5	Blank[c]	202	0.025 ± 0.002	-

[a] The *P* value was compared to the background values obtained in group 4 or 5.

[b] Background value of *E. histolytica* positive feces in control plates in which either policlonal or monoclonal antibody was not used.

[c] Background values from the 202 *E. histolytica*-free fecal samples.

Table III

Determination of the Presence of *E. histolytica* in Feces by Microscopy and by ELISA[a]

Parasite	Microscopy	ELISA
None	213/429	0
E. histolytica	13[b]/429	26[c]/429
Other parasites:	216/429	(0/216)
E. coli	29	0
G. lamblia	57	0
E. nana	59	0
I. butschlii	9	0
Ch. mesnilii	8	0
A. lumbricoides	30	0
T. trichura	1	0

[a] The sample for the ELISA assay was taken without weighing the feces

[b] Thirteen of the 26 found positive by ELISA and no others

[c] Some samples contained more than one parasite

FIGURE LEGENDS

Fig. 1 Sensitivity of the ELISA. Points indicate reactivity of the monoclonal anti-*E. histolytica* against E. histolytica membrane antigen (A) or trophozoites (B).

Fig. 2 Range of reactivity of the ELISA in *E. histolytica* positive and negative human fecal samples used at the concentrations shown on the abscissa.

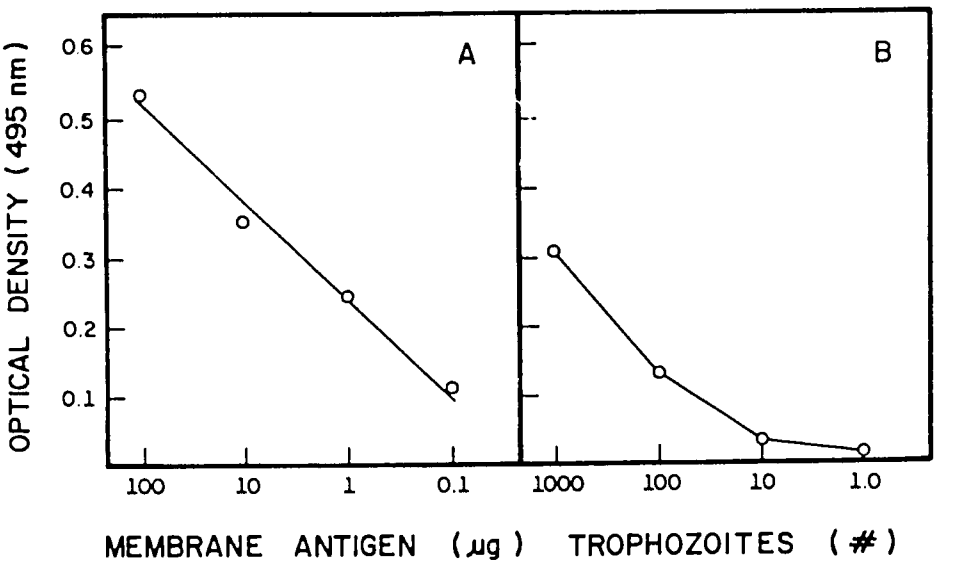
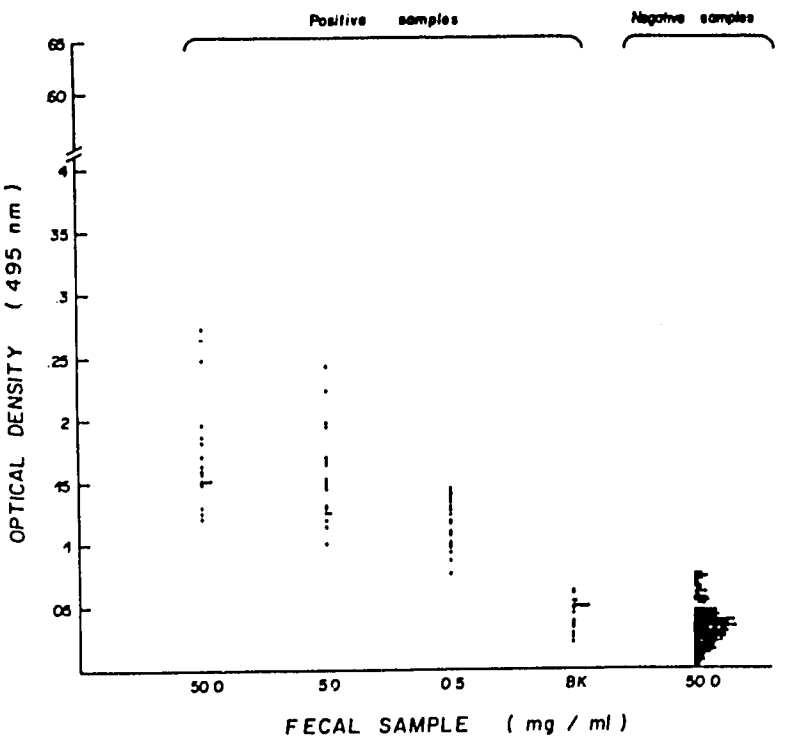

OPTICAL DENSITY (495 nm)
0.6
0.5
0.4
0.3
0.2
0.1
A
B
100
10
1
0.1
1000
100
10
1.0
MEMBRANE ANTIGEN (µg)
TROPHOZOITES (#)

Fig. 1


Positive samples
Negative samples
OPTICAL DENSITY (495 nm)
65
60
4
3.5
3
2.5
2
1.5
1
0.5
50 0
5 0
0 5
BK
50 0
FECAL SAMPLE (mg / ml)

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efecto del acoplamiento de un activador de la vía alterna del complemento a un anticuerpo monoclonal anti-*Entamoeba histolytica* no activador del complemento

Resumen

En este estudio se informa el efecto que presenta el complejo de anticuerpo monoclonal anti-*E. histolytica* (MNC) - factor del veneno de cobra (FVC) sobre los trofozoitos de *Entamoeba histolytica*. Los resultados que aquí se presentan indican que el complejo MNC-FVC ejerce un efecto citopatogénico (ECP) sobre amibas en cultivos, el cual puede ser de utilidad en la eliminación selectiva de éstas.

Abstract

This paper reports the effects produced by a complex of anti-*E. histolytica* monoclonal antibody complexed with cobra venom factor on *E. histolytica* trophozoites. The results show that this covalent complex exested a cytopathogenie effect (ECP) on amebie growth in culture media, which can be useful for a selective elimination of them.

El acoplamiento de anticuerpos monoclonales que tienen como característica ser altamente específicos con sustancias citotóxicas, puede resultar en un método efectivo para eliminar selectivamente poblaciones celulares definidas. En la literatura encontramos informes en los cuales se han unido covalentemente moléculas de anticuerpo a diversos agentes, como la toxina diftérica, y el ricino.[1] Ya que el complemento sérico ejerce una actividad citopatogénica sobre la *Entamoeba histolytica*,[2,3] es de pensarse que podría ser interesante conjugar un anticuerpo monoclonal específico para *E. histolytica* que *per se* no activa el complemento, con un activador del complemento y determinar si este complejo es capaz de destruir trofozoítos de *E. histolytica*. En el presente informe, se refiere el efecto que el complejo anticuerpo monoclonal anti-*E. histolytica* (MNC) - Factor veneno de cobra (FVC) posee sobre los trofozoítos de *E. histolytica* en la presencia de una fuente de complemento sérico.

Material y métodos

El anticuerpo monoclonal anti-*E. histolytica* (MNC) fue obtenido por el método previamente descrito, y corresponde a una IgG3.[4]

El FVC se obtuvo inicialmente del veneno de cobra liofilizado (International Biological Extracts) de acuerdo con el método previamente descrito.[1]

La preparación del complejo FVC-MNC se realizó por la técnica descrita por Vogel y Müller-Eberhard.[1] Para ello se usó un amortiguador salino de fosfatos 0.1 M, pH 7.4, que contenía 20 mM de N-succinimidil-3-(2-piridilditio) propionato (Pharmacia) en etanol. La mezcla se agitó a 20°C durante 30 min y se filtró a través de una columna de Sephadex G-25 para eliminar la N-hidroxisuccinimida liberada y el exceso de reactivos. Este procedimiento formó grupos 2-piridilditiopropionilo unidos al FVC o al MNC.

A una solución de MNC 2-piridilditiopropionilado en amortiguador de acetatos 0.1M en NaCl 0.1M, de pH 4.5, se le adicionó 500 mM de ditiotreitol para alcanzar una concentración de 45 mM. La mezcla de agitó a 20°C por 20 min y después se filtró a través de Sephadex G-25 para eliminar la piridina-2-tiona liberada y el exceso de ditiotreitol. El MNC que contenía grupos tiol se usó para el acoplamiento. Con este propósito se mezclaron cantidades equimoleculares del FVC 2-piridil-ditiopropionilado y el MNC tiolado a 20° C durante 21 h en 3 ml a una concentración de 1.5 mg de proteína por ml y después se filtró a 4° C en una columna de Ultrogel AcA 22 (LKB, 1.6 x 56 cm). Se colectaron fracciones de 1.2-ml a un flujo lineal de 2 cm/h, para separar la piridina-2-tiona liberada y el FVC y MNC que no reaccionaron del complejo FVC-MNC formado.

Se usaron trofozoítos de *E. histolytica* de la cepa HM1:IMSS, cultivadas por 72 h en medio de Diamond.[5]

El efecto de FVC-MNC sobre las amibas se determinó centrifugando estos cultivos a 1000 rpm durante 5 min; los paquetes obtenidos se lavaron dos veces con amortiguador de fosfatos isotónico 0.01 M, pH 7.2, ajustando los trofozoítos finalmente a 18500 por 0.1 ml de medio de cultivo, los cuales se agregaron a tubos para cultivo. De esta manera se hicieron cuatro grupos: (a) A las amibas ya ajustadas se les agregó 50 μl del complejo (7.5 μg) FVC-MNC y se incubaron a 4° C durante 60 min; se lavaron dos veces por centrifugación a 1000 rpm y se resuspendieron en 13 ml de medio de cultivo de Diamond que contenía suero fresco de cobayo como fuente de complemento, a una dilución final de 1:8 y se incubaron a 37° C por espacio de tres días. (b) Amibas tratadas con 50 μl del complejo FVC-MNC (7.5 μg); (c) Amibas a las que se les adicionaron 13 ml del medio que contenía suero de cobayo fresco a una dilución final de 1:8, y (d) Tubos testigo que contenían exclusivamente amibas en ml del medio de Diamond.

El número de trofozoítos de cada cultivo se determinó a las 24, 48 y 72 h en cámara de Neubauer.

Resultados

En la figura 1 se presenta el aislamiento del complejo MNC-FVC obtenido por filtración en gel. En ella se muestran las fracciones obtenidas y aquélla que se utilizó para este estudio, correspondiente a los tubos del 60 al 85.

En aquellos cultivos de trofozoítos en donde se adicionó el complejo MNC-FVC, más el complemento en

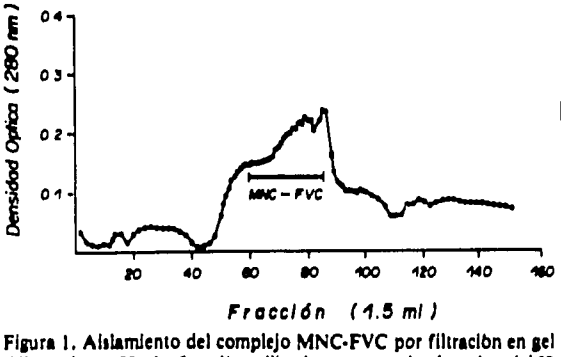

Figura 1. Aislamiento del complejo MNC-FVC por filtración en gel (Ultrogel AcA 22). La fracción utilizada corresponde a los tubos del 60 al 85.

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dilución 1:8, se observa una marcada inhibición en el crecimiento exponencial en el número de amibas, efecto que aumentó a lo largo de las observaciones, siendo mayor a las 72 h (cuadro I). Los testigos que contenían el MNC o el complemento, sólo, ejercieron cierta actividad inhibitoria, sin embargo el efecto fue mucho menor que el observado en presencia del complejo MNC-FVC.

CUADRO I. EFECTO DEL MNC-FVC SOBRE LOS TROFOZOITOS DE *E. HSITOLYTICA*

Amibas cultivadas en presencia de:	Número de trofozoítos × 10^{-3}			
	0 h	24 h	48 h	72 h
Medio	150	360	780	1 560
complemento 1:8	160	380	730	750
MNC-FVC	150	165	370	430
MNC-FVC + complemento	150	188	200	50

Estos resultados representan el promedio de 5 experimentos

Discusión

En este informe se demuestra que el complejo covalente MNC-FVC, en presencia de complemento, ejerce un ECP sobre los trofozoítos de *E. histolytica*. Este efecto es mucho mayor que el observado cuando uno de los componentes del complejo se añaden por separado a los cultivos de amibas.

La molécula efectora (FVC) utilizada se combina con el factor B de los mamíferos, correspondiente a la principal proenzima de la vía alterna del complemento, lo cual activa al factor D formando la C3/C5 convertasa, o FVC,Bb.[6] Esta enzima es muy parecida fisiológicamente a la enzima C3b,Bb,[7] aunque su vida media es mucho mayor, 7 h;[6] además, el complejo FVC,Bb es completamente resistente a la acción de las proteínas reguladoras del complemento, que actúan sobre C3b,Bb y que degradan a C3b. La enzima dependiente del FVC puede activar directamente a C5 e iniciar la formación del complejo que ataca a la membrana sin la presencia de C3.[1]

Desconocemos el efecto de este complejo *in vivo*; sin embargo, en otros estudios se ha demostrado que la vida media del FVC en circulación es de 32 h[8] y que después de 4 días se establece una respuesta inmune contra el FVC que lo eliminan. Este complejo puede ser útil para la eliminación selectiva de trofozoítos que presenten los epitopes correspondientes y de esta forma se pudieran seleccionar amibas con diferentes determinantes antigénicos.

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interacción de *Entamoeba histolytica* y anticuerpos *in situ*

El papel de la inmunidad humoral en la amibiasis invasora ha sido estudiada en sistemas *in vitro*. Se ha descrito el efecto citopatogénico del anticuerpo sobre el trofozoíto de *E. histolytica*[1-3] y la activación de la vía clásica y alterna del complemento por las amibas;[4-7] sin embargo, se desconoce el papel que tales anticuerpos puedan tener *in vivo*. Con objeto de iniciar estos estudios decidimos investigar si el anticuerpo presente en individuos con absceso hepático amibiano interacciona con los trofozoítos que se localizan en el hígado. Con tal propósito se investigó en hámsters inoculados intrahepáticamente con *E. histolytica* la presencia de estos componentes a diferentes intervalos de inoculación por el uso de anticuerpos específicos fluorescentes.

Material y métodos

Se utilizaron hámsters dorados de Siria, de ambos sexos, de 7 a 10 días de nacidos, cada uno de los cuales se infectó intrahepáticamente con 35000 trofozoítos de *Entamoeba histolytica* de la cepa HMI:IMSS, provenientes de cultivos axénicos[8] de 72 horas de desarrollo. Las dosis se administraron en volúmenes de 0.08 ml en medio de cultivo y los animales se sacrificaron a los 3, 5, 8, 15, 19 y 51 días después de la inoculación.

Cada experimento consistió de cuatro animales tomados al azar. De cada uno se obtuvo la sangre para la posterior cuantificación de anticuerpos anti *E. histolytica* por contrainmunoelectroforesis (CIE)[9,10] y hemaglutinación indirecta (HAI)[11] y el hígado para valorar la presencia de abscesos micro o macroscópicos.[12] De las porciones de hígado que presentaban indicios de lesión o abscesos francos se hicieron fragmentos de 0.5 cm^3 que se fijaron en formol al 10 por ciento, se incluyeron en parafina; de éstos se hicieron cortes a distintos niveles para detectar sobre las amibas presentes en ellos, tanto inmunoglobulinas del hospedero como aquellos determinantes antigénicos que regularmente poseen en medio de cultivo. Para poner de manifiesto la inmunoglobulina del hospedero se utilizó un antihámster (Cappel, Malvern, PA), marcado con rodamina a una dilución 1:20. La reacción se incubó por 2 horas seguida de dos lavados con solución salina isotónica (SSI), para finalmente ser observados al microscopio con epifluorescencia.

Para la detección de los determ antes antigénicos se utilizó un anticuerpo monoclor le la clase IgG, que a su vez se evidenció con el anti ro correspondiente, marcado con isotiocianato de fluoresceína (FITC), en dilución 1:20. Los cortes se incubaron con el monoclonal por espacio de 1 hora, transcurrida la cual se lavaron dos veces con SSI. Enseguida se adicionó el conjugado que se dejó actuar por una hora más, se lavó nuevamente con SSI y se observó con epifluorescencia.

En todos los casos se usaron como patrones de comparación amibas de medio de cultivo axénico de la misma cepa, procesadas de igual manera. La evaluación de la inmunoglobulina del hospedero sobre las amibas, así como de los determinantes antigénicos se efectuó por la observación de cortes a diferentes niveles de cada lesión, sacando el porciento de amibas marcadas en cada uno de ellos.

Resultados y Discusión

En los animales de 5 días de evolución no se detectaron anticuerpos antiamiba ni por CIE ni por IHA, así como tampoco inmunoglobulinas del hospedero sobre las amibas halladas en los cortes (figura 1). Esto es fácil

de entender si consideramos lo incipiente de la respuesta primaria.

En los animales de 8 días subsecuentes a la infección, los títulos de anticuerpos fueron variables, siendo algunas veces en un mismo suero, positivos por CIE y negativos por IHA o viceversa; sin embargo, cuando presentes, los títulos siempre fueron bajos (1:2). En algunos animales donde la CIE resultó positiva se llegaron a encontrar amibas que al ser marcadas con el antihámster rodamina mostraron un puntilleo fino fluorescentre, ocasionado porque el anticuerpo del hospedero comienza a interaccionar con ellas (figura 2). Cuando se usó el monoclonal IgG casi el total de las amibas resultaron marcadas. Este monoclonal también se depositó sobre una gran cantidad de antígeno, producto de la destrucción celular.

A partir del día 15, todos los sueros tuvieron un aumento en los niveles de anticuerpo de 1:8 a 1:32 por IHA y de 1:16 hasta 1:512 por CIE. Del 95 al 100 por ciento de las amibas tenían inmunoglobulina del hospedero y en su superficie (figuras 3 y 4) el monoclonal marcó sólo del 10 al 25 por ciento de ellas. Esta disminución respecto a los grupos menos evolucionados pudiera estar ocasionada porque a los quince días ya se ha establecido la respuesta secundaria y los anticuerpos del hospedero se encuentran interaccionando con estos receptores.

Tanto en los grupos de 19 como de 51 días los niveles de anticuerpos se mantuvieron elevados. En muchos de estos animales los abscesos son grandes y extrahepáticos con la aparición de líquido de ascitis, el cual también mostró tener títulos altos de anticuerpo. Aquí, las amibas interaccionan de manera muy variable con la inmunoglobulina del hospedero. A este respecto podemos suponer que el antígeno resultante de la destrucción de las amibas llega a bloquear o a interceptar la reacción con las amibas íntegras y a esto se deba el que la inmunoglobulina del animal no se detecte de manera uniforme. En los animales tanto de 19 como de 51 días, el monoclonal IgG marcó del 90 al 100 por ciento de ellos, como también a una gran cantidad de antígeno disperso (figuras 5 y 6).

Conclusiones

Los resultados que aquí se presentan, indican que después de la inoculación intrahepática de *E. histolytica*, hay una respuesta humoral con la aparición de anticuerpos séricos después de los 8 días de evolución en parte de los animales tratados. Sin embargo, a partir del día 15 todos los animales presentaron anticuerpos circulantes. La presencia de estos anticuerpos, correlaciona con el depósito de ellos sobre la superficie de las amibas en los cortes de absceso hepático amibiano. El anticuerpo monoclonal específico anti *E. histolytica* no reaccionó o lo hizo en baja proporción, con las amibas del absceso, cuando el anticuerpo del hospedero estaba presente, indicando que sus determinantes antigénicos se encuentran bloqueados por el anticuerpo del propio animal. En algunos casos donde se observa destrucción de las amibas, se visualiza una fluorescencia marcada entre el anticuerpo monoclonal y los detritos, y las amibas que llegan a encontrarse en esta zona no presentan fluorescencia.

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efecto de sobrenadantes obtenidos de linfocitos estimulados con concanavalina A sobre trofozoítos de *Entamoeba histolytica**

La participación de la inmunidad celular durante la infección por *Entamoeba histolytica* ha sido demostrada tanto *in vitro* como *in vivo*.[1] Sin embargo, su contribución no ha sido demostrada. Con este propósito se han realizado numerosos estudios en los cuales animales de experimentación han sido tratados con drogas inmunosupresoras o bien por extirpación de órganos linfoides primarios y secundarios, observándose en todos los casos que tales procedimientos disminuyen la resistencia del hospedero a la infección amibiana.[2-5] Estudios de citotoxicidad directa tendientes a definir el papel de la inmunidad celular en la amibiasis han sido realizados por varios investigadores. Así, se ha observado que cuando los trofozoítos de *E. histolytica* son confrontados con linfocitos periféricos de pacientes con absceso hepático amibiano, durante las etapas iniciales del absceso, las amibas fagocitan a los linfocitos; por el contrario, en la etapa de recuperación de la enfermedad, los linfocitos matan a las amibas.[6] Resultados similares se obtuvieron en experimentos realizados en hámsters, donde se encontró que mientras los animales inmunizados destruían a los trofozoítos de *E. histolytica*, los animales infectados no mostraban un efecto citotóxico significativo sobre el parásito.[7]

Recientemente se ha demostrado que los macrófagos humanos activados y también linfocitos T citotóxicos ejercen un efecto letal significativo sobre *E. histolytica* después del contacto directo.[8] Asimismo, se ha observado tanto *in vitro* como *in vivo* que las células de exudado peritoneal y los linfocitos de ganglios linfáticos mesentéricos de jerbo con amibiasis cecal o aquellos inmunizados con extractos de amibas, ejercen un efecto citopatogénico mayor sobre amibas no patógenas, que en aquellas cepas patógenas.[9] Es posible que la inmunidad celular participe en la resistencia a la infección a través de la acción de mediadores solubles o linfocinas, ya que la citotoxicidad mediatizada por linfocitos T es restringida genéticamente.[10] Con este objetivo, decidimos estudiar el efecto de dichas linfocinas, para lo cual se usaron los sobrenadantes de cultivos de células linfoides estimuladas *in vitro* con concanavalina A (Con-A), una lectina que favorece la formación de estos mediadores.[11] Los resultados aquí descritos indican que las linfocinas así obtenidas ejercen un efecto inhibidor en el crecimiento de *E. histolytica*.

Materiales y métodos

Animales. Se usaron ratas Wistar, hembras, de dos a tres meses de edad, procedentes del bioterio del Instituto de Investigaciones Biomédicas, UNAM. Las ratas se alimentaron *ad libitum* con tabletas de purina (Purina de México, S.A. de C.V.) y agua clorinada.

Trofozoítos. Se usaron trofozoítos de *E. histolytica* de la cepa HM1:IMSS en fase logarítmica. Esta cepa se mantuvo en cultivos desarrollados en medio axénico.[12] dio axénico.[12]

Preparación de la suspensión celular. Se obtuvieron células de bazo de ratas Wistar. La suspensión celular se preparó quitando la cápsula del bazo y homogeneizando suavemente las células en solución salina balanceada de Hanks (SSB). La suspensión celular se dejó reposar 3 min y la parte superior del sobrenadante se pasó a otro tubo, dejando solamente la parte inferior del mismo que contenía agregados celulares grandes. El sobrenadante libre de agregados se centrifugó a 200 × G, y el paquete de células se resuspendió en medio de cultivo RPMI-1640 suplementado con 5×10^{-5} de 2-mercaptoetanol, 1 mM de glutamina, 100 U/ml de penicilina, 100 μg/ml de estreptomicina y suero fetal bovino inactivado previamente, a una concentración final del 10 por ciento. La viabilidad celular se determinó por la exclusión del azul tripano y la concentración de las mismas se determinó en una cámara cuentaglóbulos.[12]

Preparación de linfocinas. Se siguió el método de preparación previamente descrito por Ortiz-Ortiz y Weigle[13] con algunas modificaciones. Esencialmente el método consiste en cultivar las células de bazo a una concentración de 5×10^6 células/ml en presencia

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de Con-A (10 μg/ml) en medio RPMI-1640 suplementado. Las células se incubaron durante 48 h a 37°C en 5 por ciento CO_2. Después de la incubación, se colectaron los sobrenadantes por centrifugación a 1000 X G durante 20 min. y se adicionó α-D-metil manosa 0.1 M final. Paralelamente se realizaron estudios de proliferación celular que sirvieran de control de la lectina.

Ensayos de proliferación celular. Las células de bazo ajustadas a 5 × 10^6 células/ml en medio RPMI-1640 suplementado se colocaron por quintuplicado y en volúmenes de 0.1 ml en placas de microtitulación (Costar). A continuación se añadió 0.1 ml de Con-A a una concentración de 10 μg/ml. Las células se incubaron en las condiciones arriba descritas y 1 μCi de ^{3}H-timidina (^{3}H-TdR, actividad específica 5.0 Ci/mmol, New England Nuclear, Boston, MA) se adicionó a cada pozo 18 h antes del ensayo. Las células se colectaron con un cosechador (Brandel, Rockville, MD); los filtros de fibra de vidrio se incorporaron en líquido de centelleo para su cuenta radioactiva.[14]

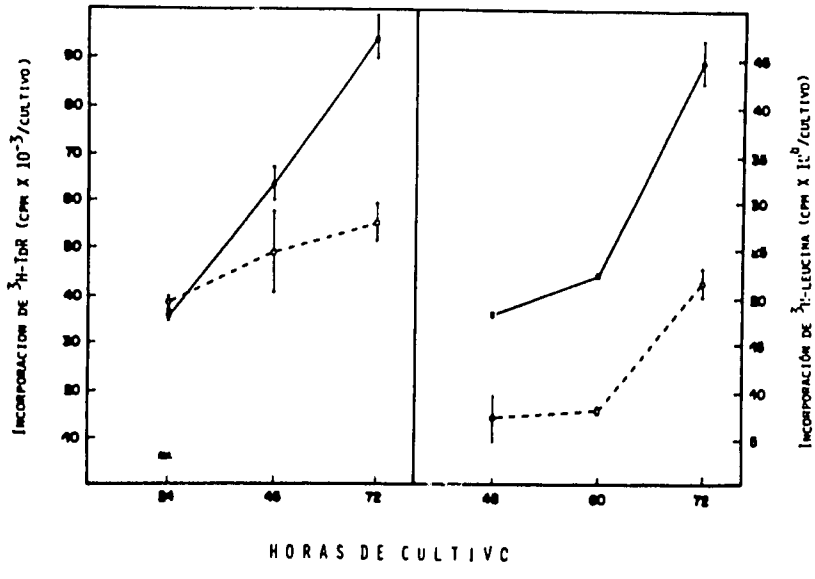
Ensayo para determinar el efecto de las linfocinas sobre los trofozoitos de E. histolytica. Para determinar el efecto de las linfocinas sobre los cultivos de *E. histolytica,* se adicionaron 4 ml del sobrenadante activado a 9 ml del cultivo de amibas en fase exponencial. Los tubos se incubaron a 35°C y se realizaron estudios de viabilidad celular a diferentes tiempos.

El efecto de las linfocinas sobre la síntesis de proteínas de la amiba se determinó por la adición de 0.5 μCi de ^{3}H-leucina (actividad específica 142 Ci/mmole; Amersham, UK) a los cultivos 12 h antes de la cosecha de los trofozoitos.[15] Los trofozoitos se cosecharon a las 48, 60 y 72 h, se lavaron tres veces con un amortiguador de fosfatos 0.14 M, de pH 7.2, por centrifugación a 200 X G por min; el sedimento se resuspendió en 1 ml de PBS que contenía 5 mM de fenilmetilsulfonil fluoruro (Sigma, Saint Louis, MO), 5 mM de metil maleimida (Sigma), y 1 mM de etildiaminotetracetato de sodio. Las suspensiones se congelaron y descongelaron cinco veces y una alícuota de 100 μl se trató con 0.5 ml de ácido tricloroacético al 25% y 0.5 ml de Casamino ácidos (Difco, Detroit, MI). Las suspensiones se incubaron durante 30 min a 4°C y después se filtraron a través de filtros de fibra de vidrio que posteriormente se secaron y contaron en un aparato de centelleo.

El efecto de las linfocinas sobre la síntesis de DNA de la amiba se determinó por la adición de 15 μCi de ^{3}H-timidina (^{3}H-TdR, actividad específica 20.0 Ci/mmole, New England Nuclear) a los cultivos de *E. histolytica* 18 h antes de su cosecha. Las amibas se colectaron a las 24, 48 y 72 h de incubación en filtros de fibra de vidrio en un cosechador (Brandel); los filtros se lavaron con agua corriente, se secaron y después se incorporaron en líquidos de centelleo para su determinación radiométrica.

Resultados

Efecto de los sobrenadantes activados sobre la viabilidad de E. histolytica. Se determinó la viabilidad y el número de amibas desde el inicio hasta la terminación del experimento. Las determinaciones se realizaron cada 24 h. En el cuadro I se muestra que los sobrenadantes activados no ejercen un efecto citopatogénico sobre los trofozoitos de *E. histolytica.* La viabilidad siempre fue superior al 90 por ciento. Por otra parte, el número de amibas en los cultivos que contenían las linfocinas se mantuvo sin cambios sig-



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nificativos durante las primeras 48 horas; sin embargo a las 72 h el número de amibas en el cultivo que contenía las linfocinas era significativamente menor que el observado en el cultivo control estimulado con un sobrenadante no activado (sin Con-A).

Efecto de los sobrenadantes activados sobre la síntesis de proteínas. Los experimentos realizados para determinar el efecto de los sobrenadantes en estudio sobre la síntesis de proteínas de *E. histolytica*, mostraron que estos inhibían significativamente la síntesis de proteínas desde las primeras 48 h de cultivo. Este efecto se mantuvo las 72 h de duración del experimento.

Efecto de los sobrenadantes activados sobre la síntesis de DNA. Con respecto a la síntesis del DNA por los trofozoítos de *E. histolytica*, ésta no se ve afectada durante las primeras 48 h; pero disminuye significativamente a las 72 h de incubación. Nuevamente, los sobrenadantes no activados no mostraron ningun efecto sobre la síntesis del DNA del parásito.

Discusión

Los resultados aquí descritos indican que las células linfoides provenientes de ratas pueden ser estimuladas en forma inespecífica, con Con-A, con la liberación de factores solubles o linfocinas que inhiben el crecimiento de trofozoítos de *E. histolytica*. Los controles confirman que la inhibición del crecimiento no es debida al consumo de los componentes del medio de cultivo. Por otra parte, la inhibición del crecimiento del protozoario no puede ser atribuida a lisis por la acción de factores tóxicos solubles, ya que no se observó disminución en la viabilidad del parásito en el transcurso del experimento. En cambio, los datos si sugieren que los sobrenadantes actúan interfiriendo con el metabolismo celular de la amiba, como lo demostró la disminución significativa en la incorporación de un precursor protéico, la ^{3}H-leucina. Este efecto se pone de manifiesto a las 48 h (figura 1), tiempo en el que no se observa ninguna alteración en la síntesis del DNA. Es interesante resaltar que el efecto en la síntesis de proteínas no disminuye a niveles significativos, el número de amibas durante este periodo. Por otra parte, a las 72 h del cultivo no solamente aparece una alteración en la síntesis de proteínas, sino también en la síntesis del DNA que repercute a su vez sobre el número de amibas. Es posible que el efecto sobre el DNA sea la consecuencia de la inhibición de la síntesis de proteínas que sabemos es un requisito importante para la división celular.[15]

EFECTO DE LINFOCINAS OBTENIDAS DE CULTIVOS DE CELULAS DE BAZO ESTIMULADAS CON CONCANAVALINA A, SOBRE TROFOZOITOS DE ENTAMOEBA HISTOLYTICA

HORAS DE CULTIVO	No. DE TROFOZOITOS				P
	CONTROL	VIABILIDAD (%)	EXPERIMENTAL	VIABILIDAD (%)	
24	200 000 ± 18 000	> 90	157 300 ± 12 600	> 90	N.S.
48	413 000 ± 31 000	> 90	300 000 ± 42 013	> 90	N.S.
72	932 000 ± 79 000	> 90	431 000 ± 57 300	> 90	0.02

LOS DATOS OBTENIDOS REPRESENTAN EL PROMEDIO ± ERROR TIPO DE 8 EXPERIMENTOS

EL VALOR P SE DETERMINÓ POR LA PRUEBA I DE STUDENT.

Nuestros resultados son compatibles con un efecto inhibidor del crecimiento de *E. histolytica* debido a productos liberados por los linfocitos bajo el estímulo de la Con-A, un mitógeno específico de los linfocitos T, que propicia la liberación de linfocinas.[11] Resultados preliminares en nuestro laboratorio indican que, cuando los sobrenadantes son acidificados a un pH de 2.5, pierden su capacidad de inhibir el crecimiento de las amibas, lo que nos hace suponer que la linfocina responsable pudiera ser el interferón gamma.[18] Son necesarios más estudios para definir la participación de esta linfocina en los fenómenos de inhibición del crecimiento de la amiba aquí descritos.

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inmunogenicidad de un antígeno deslipidizado de *Entamoeba histolytica* *

Resumen

Durante el último decenio se han descrito intentos encaminados a inducir inmunidad protectora en la amibiasis. Para ello se han utilizado diversos preparados antigénicos, en su mayoría provenientes de la *Entamoeba histolytica* HM1:IMSS.[3] Ya que estos intentos han sido los pasos previos para su posible utilización en seres humanos, se decidió ensayar con antígenos que pudieran ser procesados fácilmente y almacenados en condiciones óptimas. Además se ha querido evaluar la capacidad inmunogénica de antígenos preparados con dos cepas de *E. histolytica*: HM1:IMSS y HM2:IMSS. La experiencia ha indicado que los antígenos crudos de la cepa HM1:IMSS, han mostrado ser inestables cuando se almacenan a bajas temperaturas, ya que tienden a precipitarse. Esta característica probablemente se debe a su alto contenido lipídico, por lo cual se decidió deslipidizarlos y comparar su capacidad para inducir una respuesta inmune respecto al antígeno total. Por otra parte se sabe que la presencia de lípidos podría actuar como un buen adyuvante de la respuesta inmune, la cual se vería afectada por el tratamiento deslipidizante. Con el fin de eliminar este efecto desfavorable, los antígenos se incorporaron en un adyuvante de uso común $Al(OH)_3$, y se observó si la capacidad inmunogénica se modificaba.

El objeto de este estudio consiste en determinar si el nuevo antígeno deslipidizado de *E. histolytica* posee propiedades inmunogénicas, capaces de conferir protección.

During the last decade trials have been referred to induce protective immunity in amebiasis. To that end different antigenic preparations have been used, mostly from *Entamoeba histolytica* strain HM1:IMSS.[3] Since these attemps have been a step previous to their use in humans, it was decided to use antigens which can be easily processed and stored under optimal conditions. Besides, the immunogenic capacity of antigens prepared from two strains of *E. histolytica:* HM2:IMSS, will be evaluated. Experience has shown that raw antigens of the HM1:IMSS strain, have demonstrated to be unstable when stored at low temperatures, since they tend to precipitate. This characteristic is probably due to its high lipid content, a reason why it was decided to delipidize them and compare their capacity to induce an immune responce in relation to the total antigen. On the other hand, it is know that the presence of lipids could act as a good adjuvant of the immune response, which would be affected by the treatment to remove lipids. With the object of removing this unfavorable effect, the antigens were incorporated in a commonly used adjuvant $Al(OH)_3$, and modifications of the immunogenic capacity were observed.

The object of this study was to determine if the new delipidized antigen of *E. histolytica* has immunogenic properties, capable of conferring protection.

Material y métodos

Antígeno de E. histolytica: Las amibas de la cepa HM1:IMSS se cultivaron en medio axénico TYI-S-33 de Diamond[4] y se cosecharon a las 72 h de cultivo; a continuación se lavaron tres veces con una solución amortiguadora de fosfato de potasio 19 mM adicionada con NaCl 0.27M, pH de 7.2. Los trofozoítos así tratados se liofilizaron y almacenaron a 4°C en desecación hasta el momento de ser utilizados. Un antígeno de la cepa HM2:IMSS, preparado de manera similar, fue proporcionado por el doctor B. Enders (Behringwerke, República Democrática Alemana). Para la preparación de las dosis de inmunización se pesaron cantidades iguales de antígeno preparado con cada una de las cepas y se suspendieron en solución salina 0.15 M, a continuación se cuantificó la concentración de proteínas por el método de Lowry y col.[5]

Deslipidización del antígeno: La deslipidización de 1 g de liofilizado de ambas cepas, se llevó a cabo por extracción con éter etílico en un aparato Soxhlet a 37°C durante 24 horas.

Adyuvante: Los antígenos adicionados con adyuvante se prepararon utilizando las normas de producción de vacunas del Instituto Nacional de Higiene de la Secretaría de Salud (1.7 mg de Al $(OH)_3$/mg de proteína).

Inmunización: Se utilizaron ratones de la cepa BALB/c distribuidos en ocho grupos de cinco animales, los cuales se inocularon por vía intraperitoneal en el día 0 y 7 con 100 μg de proteína por ratón.

*Detección de anticuerpos séricos anti-*E. histolytica *por el método de ELISA:* Los diferentes grupos de ratones se sangraron por punción del plexo retroorbitario en los días 7 y 14 después de la primera inoculación. El ensayo inmunoenzimático se llevó a cabo en placas cubiertas con 1 × 10^3 trofozoítos de *E. histolytica* HM1:IMSS por pozo, siguiendo el método descrito previamente.[6] La presencia de anticuerpo anti-*E. histolytica* se puso de manifiesto por medio de una anti-inmunoglobulina de ratón conjugada a la enzima peroxidasa (Cappel, Malvern, PA). Como sustrato se usó una solución de *orto*-fenilendiamina (10 mg/ml, Sigma, Saint Louis, MO) en una solución amortiguadora de citratos 0.1 M, pH 4.5. La reacción se dejó reposar durante 8 min y finalmente se bloqueó con una solución de H_2SO_4 1 M. Las placas se leyeron en un procesador de ELISA (Behring, ELISA-processor M) a una longitud de onda de 495 nm.

Estudios de protección: Para estos estudios se inmunizaron hámsters con 1 mg de proteína de antígeno deslipidizado para después ser desafiados por vía intrahepática con 100 000 trofozoítos en el día 15. Para evaluar este estudio se investigó en cada animal la presencia de anticuerpos anti-*E. histolytica* en el suero por la técnica de contrainmunoelectroforesis.[7]

Análisis estadístico: Para el análisis estadístico se utilizó la prueba *t* de Student si las varianzas eran homogéneas y la prueba de Mann-Whitney si éstas eran heterogéneas.

Resultados

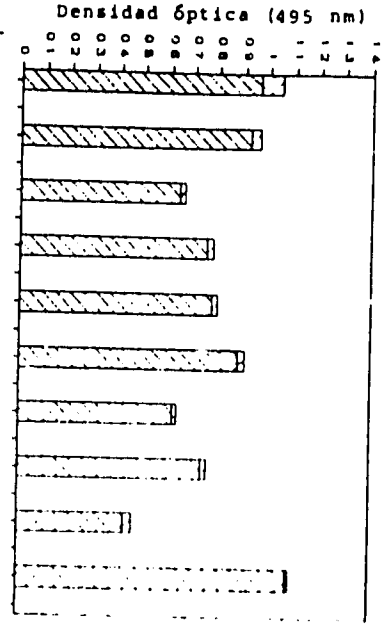
En la figura 1 se presentan los resultados de la respuesta primaria en los ratones inmunizados con los antígenos en estudio. Como se puede observar, con excepción de los grupos 1 y 2, la comparación entre los grupos inmunizados con antígeno adicionado con adyuvante (grupos 4, 6 y 8), y los inmunizados con antígeno sin adyuvante (grupos 3, 5 y 7) muestran diferencias significativas ($p < 0.05$), lo cual indica que la presencia de adyuvante mejora la respuesta humoral.

Al comparar los grupos inmunizados con el antígeno total con *E. histolytica* cepa HM2:IMSS y el inmunizado con antígeno total preparado con la cepa HM1:IMSS se encontró que la respuesta de formación de anticuerpos es en general similar, sin embargo, algunos grupos mostraron diferencias significativas en favor de la cepa HM2:IMSS (grupo 1 vs 5, $p < 0.05$). Estos mismos resultados se repitieron al comparar los grupos inoculados con antígeno deslipidizado de la cepa HM2:IMSS y de la cepa HM1:IMSS. Asimismo, no existen diferencias en la respuesta inducida por los antígenos totales o los deslipidizados administrados con adyuvante (grupo 2 vs 6 y 4 vs 8).

En la respuesta secundaria (figura 2) se observan diferencias significativas ($p < 0.05$) entre los grupos inoculados con antígeno total (grupos 1 y 5) y los inoculados con estos mismos antígenos adicionados con adyuvante (grupos 2 y 6). Cuando se compararon los grupos inoculados con antígenos deslipidizados (grupos 3 y 7) y los inoculados con los antígenos deslipidizados adicionados con adyuvante (grupos 4 y 8), no se encuentran diferencias significativas tanto para los antígenos preparados con la cepa HM2:IMSS como para la cepa HM1:IMSS. Sin embargo, si se compara la respuesta de los antígenos deslipidizados de la cepa HM2:IMSS y HM1:IMSS (grupos 3 y 7) las diferencias son significativas ($p < 0.05$). Por otra parte la respuesta inducida por los antígenos deslipidizados preparados con adyuvante (grupos 4 y 8), fue similar.

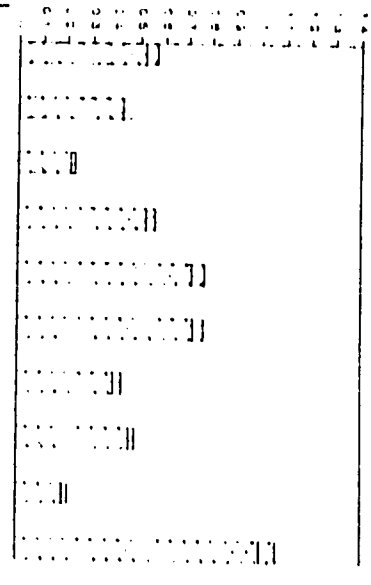
RESPUESTA PRIMARIA EN RATONES BALB/c INMUNIZADOS CON *ENTAMOEBA HISTOLYTICA*, CEPAS HM1:IMSS y HM2:IMSS

Grupo	Inmunógeno
1	HM2:IMSS NO DESLIPIDIZADA
2	HM2:IMSS NO DESLIPIDIZADA + ADYUVANTE
3	HM2:IMSS DESLIPIDIZADA
4	HM2:IMSS DESLIPIDIZADA + ADYUVANTE
5	HM1:IMSS NO DESLIPIDIZADA
6	HM1:IMSS NO DESLIPIDIZADA + ADYUVANTE
7	HM1:IMSS DESLIPIDIZADA
8	HM1:IMSS DESLIPIDIZADA + ADYUVANTE
9	NINGUNO (SUERO NORMAL)
10	NINGUNO (SUERO INMUNE ANTI-*E. HISTOLYTICA*)



RESPUESTA SECUNDARIA EN RATONES BALB/c INMUNIZADOS CON *ENTAMOEBA HISTOLYTICA*, CEPAS HM1:IMSS y HM2:IMSS

Grupo	Inmunógeno
1	HM2:IMSS NO DESLIPIDIZADA
2	HM2:IMSS NO DESLIPIDIZADA + ADYUVANTE
3	HM2:IMSS DESLIPIDIZADA
4	HM2:IMSS DESLIPIDIZADA + ADYUVANTE
5	HM1:IMSS NO DESLIPIDIZADA
6	HM1:IMSS NO DESLIPIDIZADA + ADYUVANTE
7	HM1:IMSS DESLIPIDIZADA
8	HM1:IMSS DESLIPIDIZADA + ADYUVANTE
9	NINGUNO (SUERO NORMAL)
10	NINGUNO (SUERO INMUNE ANTI-*E. HISTOLYTICA*)



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Los resultados de los experimentos de protección indican que los hámsters inmunizados con el antígeno deslipidizado, tanto de la cepa HM1:IMSS como de la cepa HM2:IMSS presentan, después de las tres dosis del antígeno inmunizante, anticuerpos en circulación. Estos animales mostraron también una resistencia al desafío amibiano como lo demostró la ausencia de abscesos en los hígados examinados. Estos resultados contrastaron con los obtenidos en los animales no inmunizados en los cuales además de la ausencia de anticuerpos se observaron abscesos macroscópicos múltiples.

Discusión

En este reporte presentamos evidencias de que un antígeno deslipidizado de *E. histolytica* es inmunogénico en los animales estudiados. Esta inmunogenicidad se reflejó tanto en la capacidad de inducir la formación de anticuerpos como en la de protección contra el desafío intrahepático con trofozoítos viables.

La reactividad cruzada entre cepas de *E. histolytica* con diferente virulencia ha sido reportada previamente;[8] apoyándose en estos resultados y dada la dificultad que ofrece el cultivo masivo de la cepa HM1:IMSS, se decidió probar dos cepas con diferentes características de cultivo y virulencia. Se ha trabajado con un extracto crudo, supuestamente más inmunogénico pero a la vez más inestable a la conservación. La deslipidización confirió mayor estabilidad, factor importante si pensamos en la preparación futura a gran escala. Este procedimiento de eliminación de lípidos indudablemente altera la inmunogenicidad del extracto, por lo cual se decidió adicionar $Al(OH)_3$ según las normas establecidas por la Secretaría de Salud. Durante la respuesta inmune primaria hubo una evidente mejoría de la respuesta, sin embargo, en el transcurso de la respuesta secundaria el papel del adyuvante no es clara.

Estos resultados por ahora preliminares han dado lugar a nuevos experimentos tales como la utilización de mayor dosis de antígeno con el fin de definir si las diferencias encontradas se confirman. También se han planeado experimentos de seguimiento de la respuesta inmune a largo plazo, así como la modificación del esquema de inmunización con objeto de optimizar el procedimiento.

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Traducción de ARN obtenido de *Entamoeba histolytica* y su caracterización con un anticuerpo monoclonal específico[1]

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Mendoza F, Ximénez C, Díaz de León L, Ortiz Ortiz L: *Traducción de ARN obtenido de* Entamoeba histolytica *y su caracterización con un anticuerpo monoclonal específico.* Salud Pública Méx., 1985; 27: 241-245.

Resumen: En este reporte describimos la obtención de productos de traducción de un ARN total de *E. histolytica*, en un sistema libre de células, y su reactividad con anticuerpos contra el parásito. Mientras los anticuerpos de pacientes con amibiasis invasora muestran una reactividad compleja con tales productos, el anticuerpo monoclonal específico para *E. histolytica* presenta una reactividad más definida, en la cual destacan tres componentes principales. La combinación de estas dos metodologías ofrece la posibilidad de obtener uno o varios mRNAs que puedan ser de utilidad, tanto en el diagnóstico como en la inmunoprofilaxis de esta enfermedad.

Las infecciones producidas por *Entamoeba histolytica* representan un problema de salud pública en el mundo entero, particularmente en los países en vías de desarrollo.[1] En los últimos años las investigaciones sobre diferentes aspectos de la estructura, fisiología y bioquímica de este parásito, han avanzado de una manera importante.[2] Uno de los factores que ha contribuido a tal progreso es el advenimiento de los cultivos axénicos de *E. histolytica*,[3] el cual ha permitido también el estudio de su relación con el huésped natural y el experimental.[4-6]

Otro aspecto que ha sido abordado con interés es el de la caracterización antigénica del parásito. Para tal objeto se han usado sueros inmunes de pacientes o de animales de experimentación, los cuales han puesto de manifiesto la complejidad antigénica de la amiba.[7-9] Recientemente se han usado anticuerpos monoclonales que han mostrado la presencia de antígeno(s) específico(s) de *E. histolytica*.[10]

Las técnicas de biología molecular, en combinación con la de anticuerpos monoclonales, ofrecen actualmente medios que permiten caracterizar inmunógenos potenciales en el des-

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arrollo de vacunas y reactivos de diagnóstico.[11, 12] En este reporte informamos sobre la detección de proteínas obtenidas en un sistema de traducción de ARN, libre de células, por sueros de pacientes con amibiasis hepática y por un anticuerpo monoclonal específico para *E. histolytica.*

MATERIAL Y METODOS

CULTIVO DE LOS PARASITOS

Las cepas HK9 y HM1:IMSS se cultivaron en medio axénico de Biosate-Peptona a 37 °C como es descrito por Diamond.[3] Los trofozoítos se cosecharon a diferentes tiempos de cultivo.

ANTICUERPOS

Se usaron anticuerpos policlonales humanos obtenidos de pacientes con amibiasis invasora y un anticuerpo monoclonal específicos para *E. histolytica* (clona MC004).

EXTRACCION DEL ARN TOTAL

El ARN se aisló de trofozoítos recién cosechados por medio del método de clorhidrato de guanidina[13] con algunas modificaciones.[14] Aproximadamente 50×10^6 trofozoítos se solubilizaron en 10 ml de solución de guanidina-HCl 8M y acetato de potasio 10 mM, pH 5 y se homogeneizaron (3×20 seg. 10,000 rpm) en un politrón. El ARN del homogenado se precipitó con 0.5 volumen de etanol absoluto y se dejó reposar a −20 °C durante 12 a 15 h. El precipitado se disolvió en 10 ml de una solución que contenía guanidina-HCl 8M, acetato de potasio 10 mM, pH 5, EDTA 20 mM pH 7. El proceso de precipitación del ARN se repitió tres veces más, y el precipitado obtenido se disolvió en 2 ml de EDTA 20 mM y se deslipidizó con una mezcla de cloroformo: butanol (4:1, v/v). Finalmente, el ARN se precipitó con acetato de sodio 3M a −20 °C. El ARN obtenido se precipitó con acetato de potasio 0.2 M de pH 6, con dos volúmenes de etanol durante 12-16 h a −20 °C.

TRADUCCION DEL ARN PARASITO

Alícuotas del ARN total se adicionaron al lisado de reticulocitos de conejo tratado con nucleasa de micrococo, tal como lo describe Pelham y Jackson.[15] La mezcla de reacción total (15 μl) contenía: lisado (5 μl), cloruro de potasio (40 mM), acetato de potasio (100 mM), mezcla de aminoácidos sin metionina (1 mM), creatina-fosfocinasa (30 mM), HEPES (75 mM pH 7.2) y como precursor radiactivo ^{35}S-metionina (10 μCi/ensayo, actividad específica 1050 μCi/mM. La mezcla se incubó por 60 min a 30°C y la traducción se terminó al agregar 2.5 μl de R Nasa pancreática (1 mg/ml) e incubar por 15 min a 30 °C.

DETERMINACION DE LA RADIOACTIVIDAD INCORPORADA

La incorporación del isótopo se determinó midiendo la radiactividad precipitable con TCA a 10% en las diferentes alícuotas. Las muestras se mantuvieron durante 30 min a 4 °C, se filtraron en membranas de nitrocelulosa y se lavaron 3 veces con TCA a 8%. Después de secar los filtros se determinó su radioactividad, para lo cual se les colocó en viales que contenían 5 ml de líquido de centelleo y se contaron en un contador de centelleo líquido.

ELECTROFORESIS DEL GEL DE POLIACRILAMIDA

Los productos de traducción se solubilizaron en solución amortiguadora que contenía SDS 2% (p/v), 2-mercaptoetanol 2% (v/v), glicerol 20% (v/v), azul de bromofenol 0.1% (p/v) y Tris-HCl 100 mM, pH 6.8. Las muestras se pusieron a ebullición en esta mezcla durante 3 min y se corrieron en el sistema discontinuo de Laemmli[16] en geles a 6% que contenían urea.

ELECTROTRANSFERENCIA

Los productos de traducción se transfirieron según el método de Towbin[17] con algunas modificaciones. Brevemente, los productos de traducción se transfirieron a papel de nitrocelulosa usando como amortiguador de electrodo, glicina 192 mM, Tris 25 mM y metanol 20% a 400 mA durante 2 h. Después de la transferencia y con el objeto de saturar los sitios de unión a proteína remanentes, el papel de nitrocelulosa se incubó durante 2 h a 35 °C en una solución amortiguadora de fosfatos salina 0.01 M, pH 7.2, con albúmina bovina (grado RIA) 1.5%, gelatina 0.25%.

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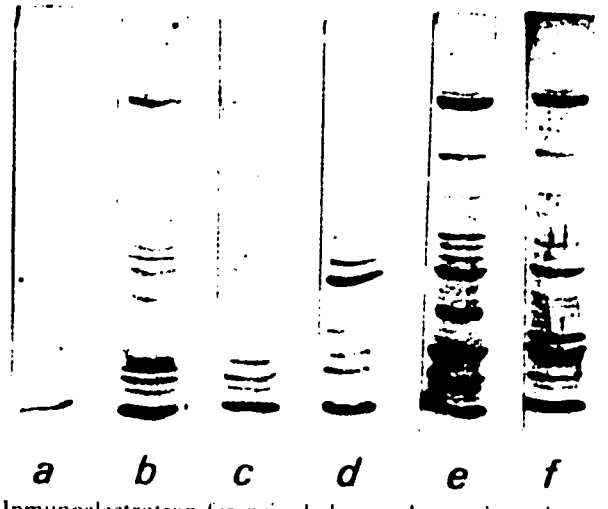
FIGURA 1

Fluorografía de los productos de traducción de ARN total obtenido de *E. histolytica* en un sistema de reticulocitos libre de células. (a) Control sin ARN exógeno; (b-f) ARN ensayado: 1.5, 3.0, 4.5, 6.0, y 7.5 μg, respectivamente.

INMUNODETECCION

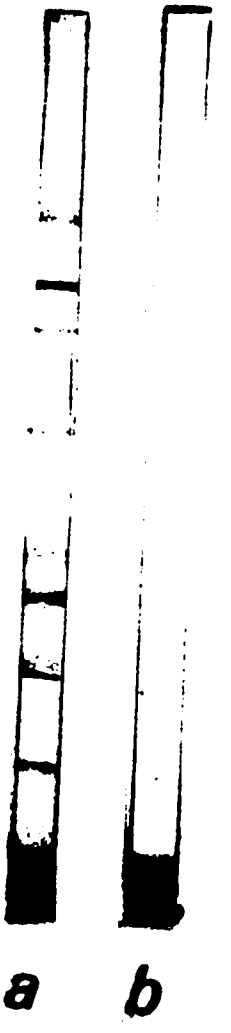
Después de bloquear la tiras de nitrocelulosa se incubaron durante 2 h con una dilución apropiada de suero de pacientes con amibiasis hepática o con el anticuerpo monoclonal específico para *E. histolytica*. Las tiras se lavaron dos veces con PBS que contenía 0.3% Tween 20 y dos veces con PBS; cada una de ellas durante 10 min. A continuación, las tiras tratadas con los sueros de pacientes se hicieron reaccionar con un anticuerpo contra las cadenas pesadas de la IgG humana y las tratadas con el anticuerpo monoclonal con proteína A. Tanto la anti-Ig humana como la proteína A se encontraban acopladas a peroxidasa. Después

FIGURA 2



Inmunoelectrotransferencia de los productos de traducción de ARN total obtenido de *E. histolytica* en un sistema de reticulocitos libre de células. El material transferido se reveló con: (a) Suero control sin anticuerpo anti-*E. histolytica*; (b-f) Sueros de pacientes con amibiasis invasora, diluidos 1:50. Para revelar se usó una anti-IgG humana acoplada a peroxidasa.

FIGURA 3



Inmunoelectrotransferencia de los productos de traducción de ARN total obtenido de *E. histolytica* en un sistema de reticulocitos libre de células. El material transferido se reveló con: (a) Anticuerpo monoclonal anti-*E. histolytica*, clona MCOO4, y (b) Suero control de ratón sin anticuerpos anti-*E. histolytica*. Para revelar se usó Proteína A-peroxidasa.

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de esta incubación, las tiras se lavaron de la manera descrita anteriormente y se revelaron con diaminobencidina (50 mg/100 ml) y peróxido de hidrógeno (100 μl de una solución que contiene 100 μl/100 ml) al 3% en PBS pH 7.2.

RESULTADOS

La figura 1 muestra la fluorografía de los productos sintetizados por el lisado de reticulocito a diferentes concentraciones de ARN. Como se puede observar hay varios componentes de pesos moleculares que varían entre 25,000 y 150,000. Cuando se obtiene ARN a diferentes tiempos de cultivo (36, 48, 60 y 72 h) no se observan cambios cualitativos ni cuantitativos en el patrón de traducción, por lo que en los experimentos posteriores se decidió extraer ARN de los trofozoítos cosechados a las 60 h de cultivo.[18]

Con respecto a los resultados de la reactividad de los sueros de pacientes con los productos de traducción, se observa un patrón complejo de reconocimiento de estos productos (Figura 2). Por otra parte, con el anticuerpo monoclonal específico para *E. histolytica* se observan esencialmente tres componentes principales (Figura 3). Los controles de IgG normal no mostraron ninguna reactividad (Figuras 2 y 3, carriles a y b respectivamente).

DISCUSION

Los anticuerpos monoclonales y policlonales contra *E. histolytica* son capaces de reconocer los productos de traducción del ARN de este parásito, obtenidos en un sistema de reticulocitos libre de células. La extracción del ARN de *E. histolytica* ha sido difícil, ya que contiene ribonucleasas resistentes a los inhibidores comúnmente usados, como la heparina, el dietilpirocarbonato y otros. Sin embargo el empleo del método de clorhidrato de guanidina[14] permitió obtener preparaciones de ARN activo.

Mientras que los sueros de pacientes con amibiasis invasora muestran un patrón complejo de reconocimiento de estos productos, el anticuerpo monocional específico para la amiba reconoce fundamentalmente tres componentes. Se observa también que el anticuerpo monoclonal reacciona, aunque con menor intensidad, con otros componentes de los productos de traducción (Figura 3). Esta reactividad múltiple que se ha observado en otros sistemas que usan anticuerpos monoclonales[11, 12, 19] puede ser debida a una síntesis independiente de proteínas con epítopes compartidos o bien artificios debidos a la degradación que puede tener lugar durante la extracción;[11, 12] aunque también se ha dicho que la proteolisis que puede ocurrir *in vivo* no tiene lugar *in vitro* y que solamente puede encontrarse el componente precursor.[20] No sabemos actualmente cuál puede ser la explicación precisa; sin embargo, en nuestro laboratorio hemos encontrado que el mismo anticuerpo monoclonal (clona MC004) muestra también tres bandas de reactividad con un antígeno de membranas de *E. histolytica* (C. Ximénez, en este mismo volumen).

La traducción del ARN en un sistema libre de células ofrece la posibilidad de aislar mensajeros de proteínas que puedan ser relevantes en protección o en diagnóstico, lo cual podría facilitar la obtención más compleja de un banco de genes. La combinación de esta técnica con la de anticuerpos monoclonales ya se ha vislumbrado[21] y nuestro interés actual es en este sentido.

Mendoza F, Ximénez C, Díaz de León L, Ortiz Ortiz L: *Translation of RNA obtained of* Entamoeba histolytica *and its characterization with a specific monoclonal antibody*. Salud Pública Méx., 1985; 27: 241-245.

Summary: We report herein on the obtaining of RNA translation products of *E. histolytica* in a cell-free system and their preliminary characterization with specific anti-*E. histolytica* antibodies. The translation products reacted both with an antisera obtained from patients with invasive amebiasis and with a monoclonal antibody specific for *E. histolytica*. Whereas a complex reactivity was observed with the former antibodies, a simpler reactivity was attained with the latter in that the monoclonal antibody recognized only three main components. The combination of these techniques may offer a possibility to obtain mRNAs that can be of importance in diagnostic or inmunoprophylaxis.

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FIG. 1

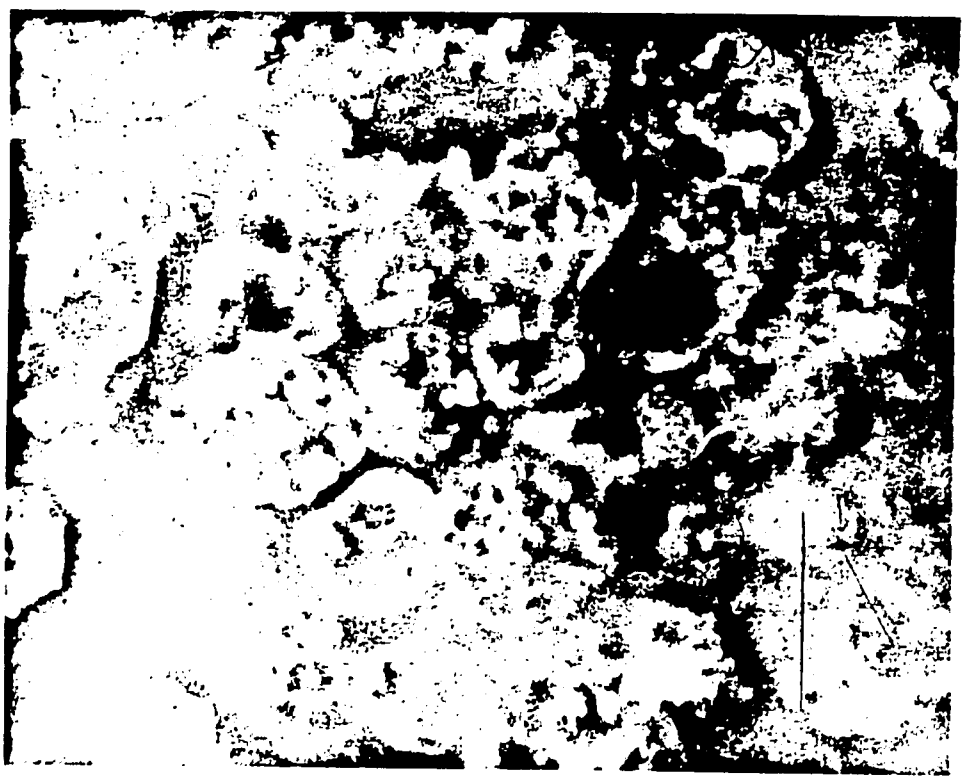

FIG. 2

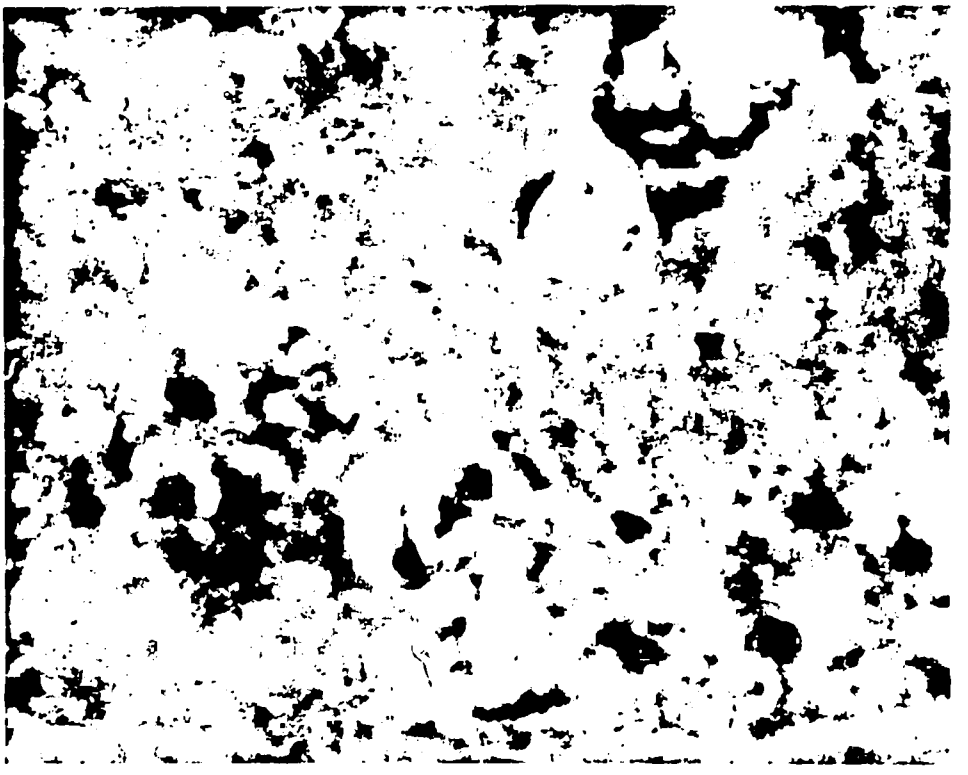

FIG. 3

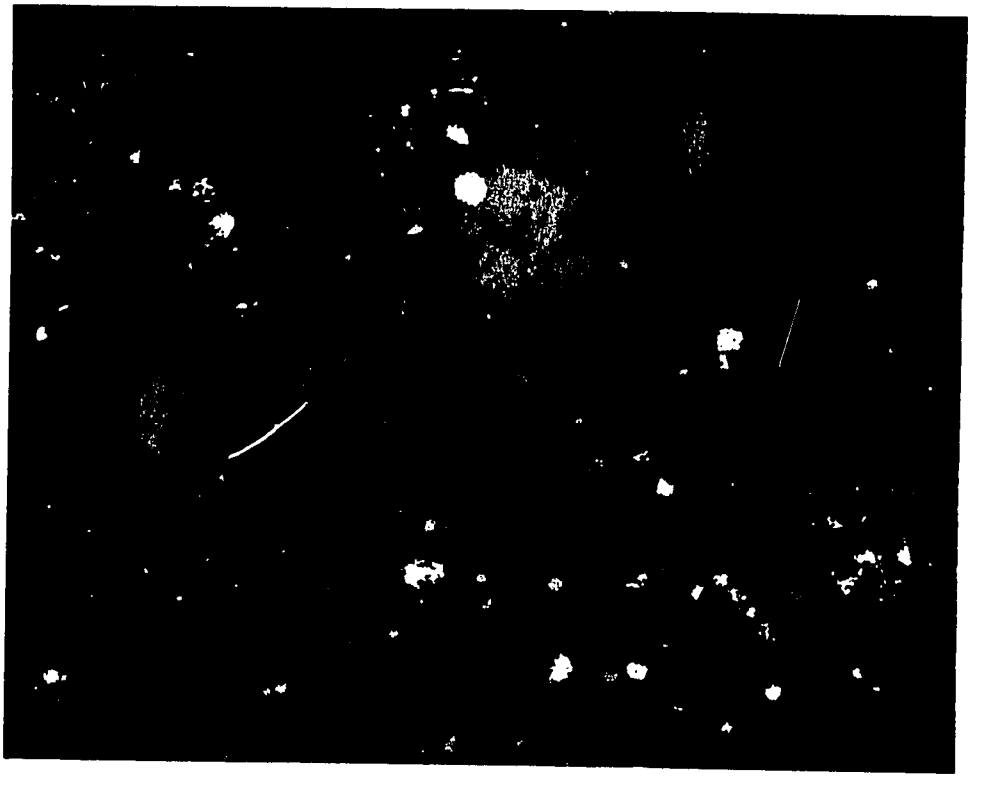

FIG. 4

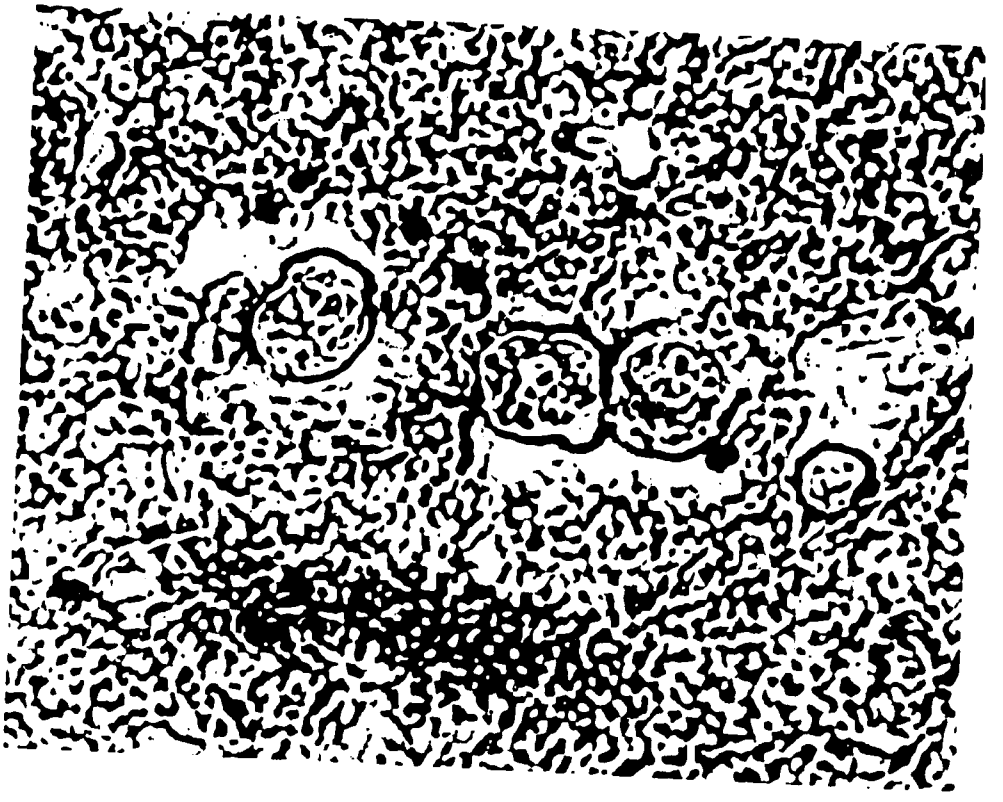

FIG. 5

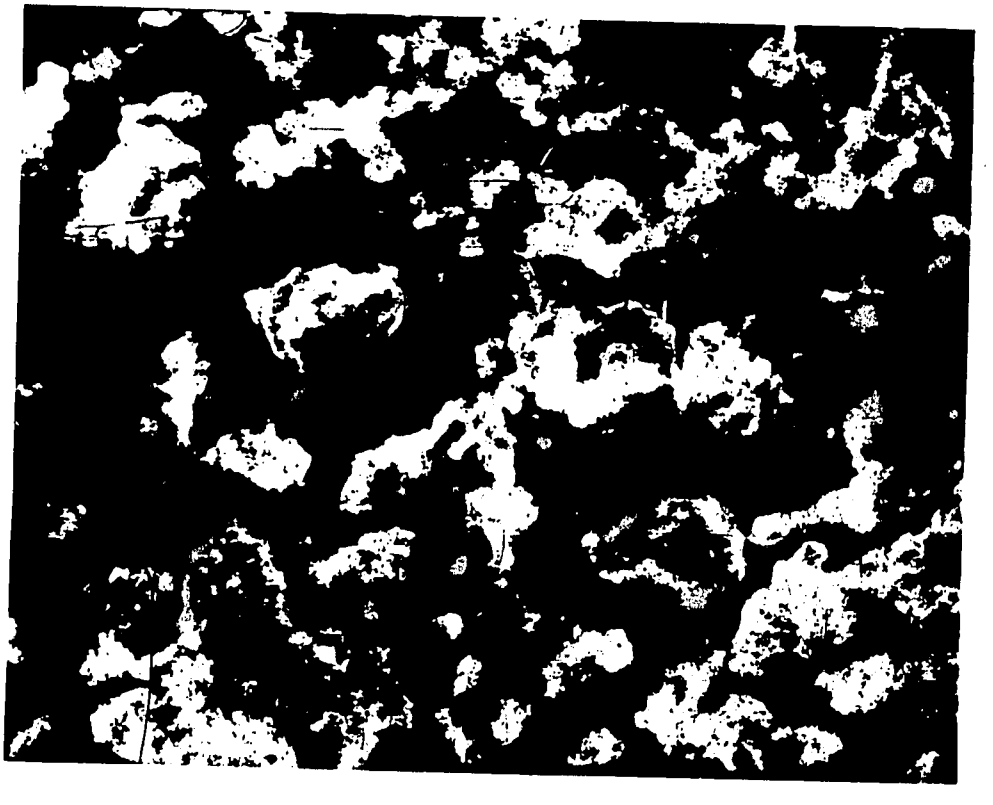

FIG. 6

Running tittle: Effect of lymphokines on *E. histolytica*.

EFFECT OF LYMPHOKINES ON THE SYNTHESES OF DNA AND PROTEINS BY *ENTAMOEBA HISTOLYTICA*[1]

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ABSTRACT

A lymphokine-rich supernatant (LRSN) and recombinant gamma interferon (IFN) inhibited the growth of *E. histolytica* trophozoites *in vitro*. These lymphokines did not show a cytotoxic effect on the ameba but rather significantly inhibited the protein and DNA syntheses of the protozoa. When the IFN had been incubated at pH 2, its effect on protein and DNA syntheses was abrocated, indicating that IFN was responsible for the observed inhibition of parasite growth. The results suggest that cell-mediated immunity may participate in protection through the activity of mediators released by lymphocytes during infection.

The participation of cellular immunity during an infection by Entamoeba histolytica has been shown both in vitro and in vivo (1). However, its role as a mechanism of resistance to amebic infection has not been demonstrated. Numerous studies have been carried out in which, it was observed that treatment of experimental animals with immunosuppresive drugs or extirpation of their primary or secondary lymphoid organs, in all cases, reduced the resistance of the host to amebic infection (2,5). With the aim of defining the role of cellular immunity in amebiasis, direct cytotoxicity studies have been carried out by various workers. Such studies have shown that, when the trophozoites of E. histolytica are confronted with peripherical lymphocytes from patients who are in the early stages of amebic liver abscess, the amebas phagocytized the lymphocytes, whereas lymphocytes from patients recovering from the abscess kill the amebas (6). Similar results were obtained in experiments carried out in hamsters since in these studies the immune system of animals immunized with ameba antigen were able to destroy the E. histolytica trophozoite, whereas infected animals were unable to mount a significant cytotoxic effect on the parasite (7).

It has recently been reported that, upon direct contact, activated human macrophages and also cytotoxic T lymphocytes exercise a significant lethal effect on E. histolytica (8,9). It has also been observed both in vitro and in vivo that peritoneal exudate cells and lymphocytes from the mesenteric lymphatic ganglia either from gerbils with cecal amebiasis or from those immunized with amebic extract exercise a greater cytopathic effect on non-pathogenic strains of ameba than on

pathogenic strains (10).

It is possible that cellular immunity participates in the resistance to amebic infection through the action of soluble mediators or lymphokines because the cytotoxicity mediated by T lymphocytes is genetically restricted (11).

With this aim, we studied the effect of said lymphokines, particularly gamma interferon (IFN) which has been implicated in the mechanism of resistance to other parasites (12, 13), on E. histolytica. In these studies, supernatants from cultures of lymphoid cells which had been stimulated in vitro with Concanavalin A (Con A), a lectin known to favor the formation of these mediators (14) and recombinant IFN were used. The results indicated that these lymphokines had and inhibitory effect on the syntheses of DNA and proteins by E. histolyica.

MATERIALS AND METHODS

Animals. Two to six month-old male Wistar rats from the Instituto de Investigaciones Biomédicas, UNAM, were used in all experiments.

E. histolytica. Seventy-two hour cultures of *E. histolytica* strain HM1:IMSS were used in all experiments. This strain was maintained in axenic culture medium (16).

Cell preparation. Cell suspension were prepared by mincing the spleen in balanced salt solution (BSS) and filtering the resulting mixtures through nylon mesh at 4°C. The cell filtrate was centrifuged at 200 X *g* and the packed cells were suspended in culture medium consisting of RPMI 1640 (Grand Island Biological Company, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum, 5 X 10^{-5} M 2-mercaptoethanol, 1 mM glutamine, 100 U penicillin/ml and 100 ug streptomycin/ml. Cell viability was determined by trypan blue exclusion and cell number was determined by hemocytometer count (17).

Preparation of lymphokine-rich supernatant (LRSN). As a source of LRSN, we used the supernatants from a culture of spleen cells which had been stimulated with Con A as described previously (17). In brief, spleen cells from Wistar rats were cultured at a density of 5 X 10^6 cells/ml in the presence of Con A (10 μg/ml) in supplemented RPMI 1640. After the cells had been incubated for 48 hr at 37°C in humidified 5% CO_2-air, the cultures were centrifuged at 10,000 X *g* for 20 min. To the resulting supernatant was added 0.1 M methyl mannoside. The cell-free supernatants were frozen at -20°C until used. Assays to determine lymphocyte proliferation (17) and

inhibition of vesicular stomatitis virus plaque formation in Vero cells (18) were run in parallel to quantitate the Con A stimulating activity. Only those LRSN which had been obtained from cells responding to Con A under the conditions previously described (17) and which had had an inhibitory effect on viral plaque formation were used. The inhibition of plaque formation by undiluted LRSN was equivalente to 1000 units of the IFN supplied by DNAX (Palo Alto, CA). Control supernatant (SN) consisted of supernatants obtained from spleen cell cultures which had not been stimulated with Con A but otherwise similarly processed.

Recombinant IFN. IFN was obtained through the generosity of Dr. Albert Zlotnik of DNAX Research Institute, Palo Alto, CA. It had been produced by transfection of murine IFN cDNA into Cos 7 monkey kidney cells. Its activity was confirmed as described previously (18). As control, we used mock-transfected supernatants of Cos 7 monkey kidney cells.

Incubation at pH 2. LRSN of IFN were dialyzed for 24 hr against 0.1 M glycine-HCl-0.15M NaCl buffer, pH 2.0, followed by dialysis for 48 hr against phosphate buffered saline, pH 7.2, and a final dialysis against complete medium. Control SN were dialyzed against PBS and complete medium.(19).

Effect of LRSN or IFN on E. histolytica trophozoites. To test the effect of LRSN or IFN on cultures of E. histolytica trophozoites, 4 ml of LRSN or 0.2 ml of a distinct number of IFN units was added to tubes containing either 9 ml (54,000 amebas) or 12.8 ml (76,800 amebas) of cultures in the exponential phase of growth (72 hr culture), respectively, to obtain a final volume of 13 ml. The tubes were incubated at 37°C and

viability determined at different times, as elsewhere described (20).

The effect of the lymphokines on the synthesis of ameba proteins was determined by adding 0.5 mCi of [^{3}H]-leucine (142 Ci/mmole; Amersham, UK) to the cultures 12 hr before harvesting the trophozoites. Trophozoites were harvested at 48, 60, and 72 hr and were washed three times with phosphate buffered saline (PBS; pH 7.2) by centrifugation at 200 X g for 10 min. The pellet was resuspended in PBS containing 5 mM phenylmethyl sulfonyl fluoride (Sigma, Saint Louis, MO), 5 mM methyl maleimide (Sigma), and 1 mM ethylendiamine-tetraacetic acid (disodium salt). The suspension was frozen and thawed five times and an aliquot of 100 µl was treated with 0.5 ml of 25% trichloroacetic followed by 0.5 ml of Casamino acids (Difco, Detroit, MI). The mixture was incubated at 4°C for 30 min and filtered through a glass fiber filter, dried, and counted in a liquid scintillation counter.(21).

The effect ot the lymphokines on the synthesis of ameba DNA was determined by adding 14 µCi of [^{3}H]-thymidine ([^{3}H]-TdR; 20.0 Ci/mmole, New England Nuclear) to the cultures 18 hr before harvesting the trophozoites. Trophozoites were harvested 24, 48, and 72 hr with a cell harvester (Brandell). The glass fiber beads were counted in a liquid scintillation counter (22).

RESULTS

Effect of LRSN on the viability and growth of E. histolytica. Culturing trophozoites with the LRSN for differente periods of time did not have any effect on the viability of the trophozoites. The viability was always greater than 90%. However, after 72 hr of incubation, the number of

ameba showed a significant decrease, attaining levels of those observed for ameba growth in the presence of control SN (Fig. 1).

Effect of LRSN on protein synthesis by *E. histolytica*. Experiments performed to determine the effect of LRSN on protein synthesis by *E. histolytica* trophozoites indicated that after 48 hr of culture, LRSN significantly inhibited the levels of protein synthesis, whereas control SN did not. This effect was maintained throughout the experiment (Fig. 2).

Effect of LRSN on DNA synthesis by *E. histolytica*. When *E. histolytica* trophozoites had been cultured in the presence of LRSN, it was observed that DNA synthesis was altered. Thus, after 48 hr of culture in the presence of LRSN, DNA synthesis began to diminish; this effect became significant after 72 hr of culture. Control SN did not show any effect on DNA synthesis in ameba cultures (Fig. 2).

Effect of pH on the inhibition of DNA synthesis by LRSN. In order to study the effect that pH may have on the inhibition of DNA synthesis by LRSN, the LRSN was adjusted to pH 2.0 for 24 hr and was thereafter readjusted to pH 7.0, as described above. Four milliliters of the LRSN thus treated was incubated in the presence of *E. histolytica* trophozoites (vida supra). The supernatants which had been maintained at pH 2.0 for 24 hr lost their capacity to inhibit DNA synthesis in *E. histolytica* trophozoites (Table I). This effect was not observed in the similarly treated control SN groups; these groups showed normal growth of amebas as compared to trophozoite growth in ameba media alone.

Effect of IFN on DNA synthesis by E. histolytica. Since the LRSN lost activity when adjusted to pH 2.0, it was of interest to determine if IFN had any effect on DNA synthesis by *E. histolytica*. IFN (20,000, 0.2 ml) incubated with *E. histolytica* trophozoites (vida supra) for 48 or 72 hr inhibited the DNA synthesis (Table II). This effect was lost when the IFN had been incubated for 24 hr at pH 2.0 (Table II). The DNA synthesis of amebas in control groups in which the supernatant medium was used was not inhibited.

DISCUSION

The results reported herein indicated that rat lymphoid cells can be stimulated non-specifically by Con A, resulting in the liberation of soluble factors or lymphokines that inhibit the growth of trophozoites of E. histolytica. The controls showed that the inhibition of growth was not due to the consumption of nutrients in the culture medium. This inhibition of ameba growth could not be attributed to lysis of the protozoa through the action of soluble toxic factors because reduced viability of the parasite throughout the course of the experiment was not observed. The data did suggest that LRSN, particularly IFN, interfered with the metabolism of the ameba as was demonstrated by the significant reduction in the incorporation of [^{3}H]-leucine. IFN was shown to be involved in this interference since, when LRSN or IFN itself were incubated at pH 2.0 for 24 hr, this inhibitory effect was abrogated.

The effect of the lymphokines was manifested at 48 hr (Fig. 1), at which time no alteration was noted in DNA synthesis. I should be noted that the effect on the synthesis of proteins did not significantly reduce the number of amebas in this period. At 72 hr of culture, an alteration was observed not only in the synthesis of proteins but also in that of DNA which, in turn, brought about a reduction in the number of amebas. It is possible that this effect on the synthesis of DNA may be a consequence of the inhibition of proteins which is known to be a prerequisite for cell division (21).

In 1975, Guerrero (6) observed that supernatants from lymphocytes obtained from individuals who had been cured of amebic liver abscess had a cytopathogenic effect on amebas. He suggested that substances liberated by lymphocytes could be cytotoxic for amebas and therefore involved in resistance through a cell-mediated immune mechanism. Our results indicated the involvement of substances distinct from those reported by Guerrero since, under our experimental conditions, we did not observe toxic effects which altered the viability of the ameba. It is interesting to note that this finding indicates that factors liberated by lymphocytes do not require contact with the ameba in order to destroy or inhibit growth of the protozoa.

IFN has been shown to be useful in other systems, for example, in the inhibition of simian malaria. IFN completely suppressed experimental infection with Plasmodium cynomologi B sporozoites in rhesus monkeys. Interestingly, this treatment had no protective effect against trophozoite-induced infections, suggesting that the IFN effect was limited to the exoerythrocytic stage of parasitic development (13).

The function of these lymphokines described herein appeared independent of their well-known activating effect on macrophages, which once activated are capable of killing amebas in axenic culture (14). This report, to our knowledge, is the first to indicate that the products liberated by lymphocytes, especially IFN, exercise an inhibitory effect on the syntheses of proteins and DNA by a parasite, in this case amebas, thereby suggesting that IFN is involved in the mechanism of resistance to infections by E. histolytica.

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TABLE I

EFFECT OF pH ON DNA SYNTHESIS BY LRSN[a]

Culture time (hr)	[^{3}H]-TdR incorporation by *E. histolytica* trophozoites (cpm/ml)					
				pH 2 incubation		
	Control SN	LRSN	*p*	Control SN	LRSN	*p*
48	7,175 ± 1,195	3,805 ± 240	< 0.02	5,700 + 455	6,315 ± 1,170	NS
72	12,000 ± 255	7,500 ± 460	< 0.01	11,505 ± 1,265	12,320 ± 1,265	NS

[a] The effect of LRSN (4 ml) on the synthesis of ameba DNA was determined by adding 15 μCi of [^{3}H]-TdR (20.0 Ci/mmole) to the cultures (54,000 trophozoites/9 ml medium) 18 hr before harvesting the trophozoites. Trophozoites were harvested 24, 48, and 72 hr with a cell harvester (Brandel). The glass fiber beads were counted in a liquid scintillation counter.

TABLE II

EFFECT OF pH ON THE INHIBITION OF DNA SYNTHESIS BY IFN [a]

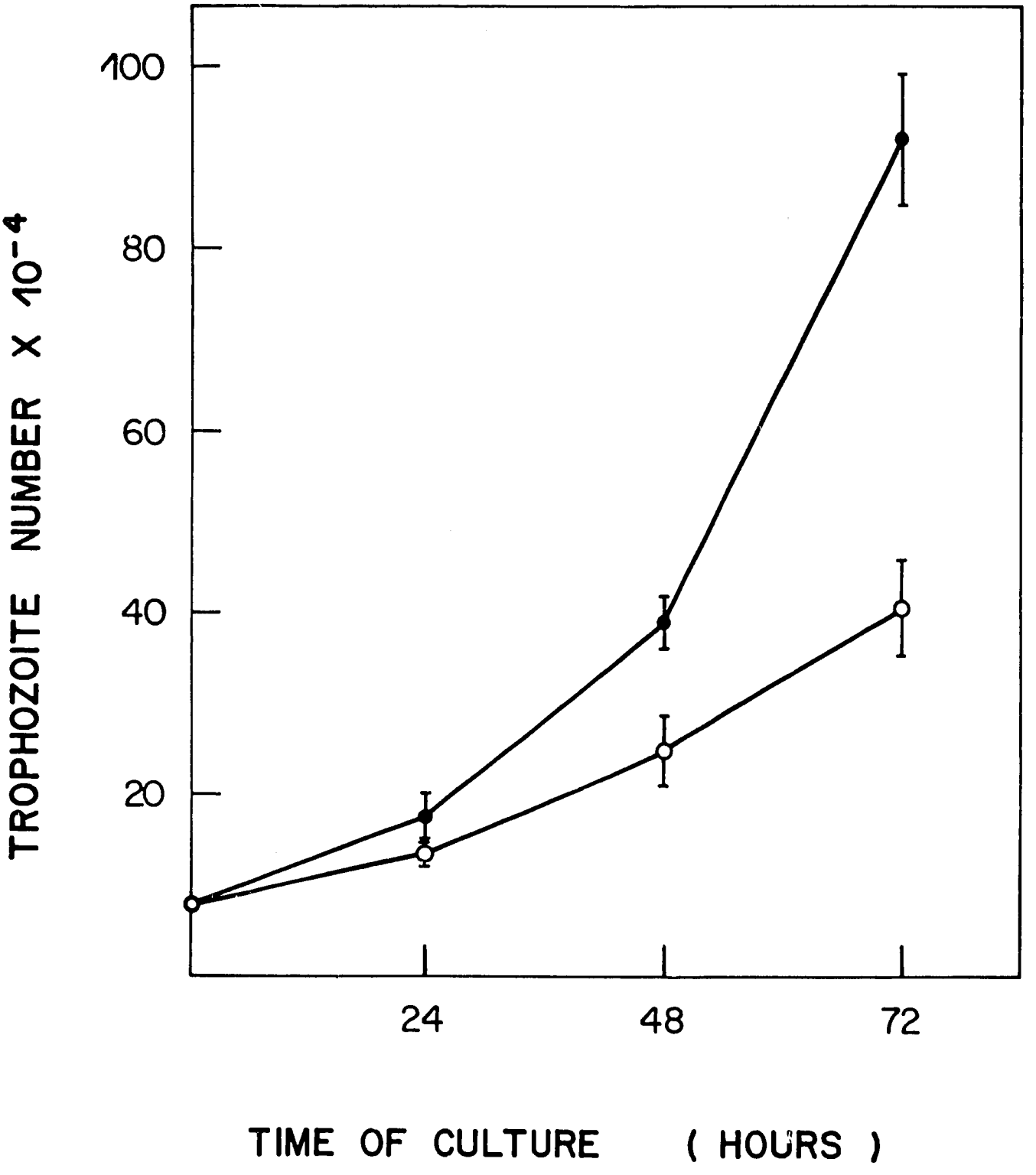
Culture time (hr)	[^{3}H]-TdR incorporation by *E. histolytica* trophozoites (cpm/ml)					
				pH 2 incubation		
	Control SN	IFN	*P*	Control SN	IFN	*P*
48	17,690 ± 542	10,513 ± 362	< 0.01	21,100 ± 198	19,500 ± 256	NS
72	36,100 ± 710	29,446 ± 1,998	< 0.01	29,950 ± 550	34,360 ± 626	NS

[a] The effect of IFN (0.2 ml) on the synthesis of ameba DNA was determined by adding 15 μCi of [^{3}H]-TdR (20.0 Ci/mmole) to the cultures (76,800 trophozoites/12.8 ml medium), 18 hr before harvesting the trophozoites. They were processed as described in Table I.

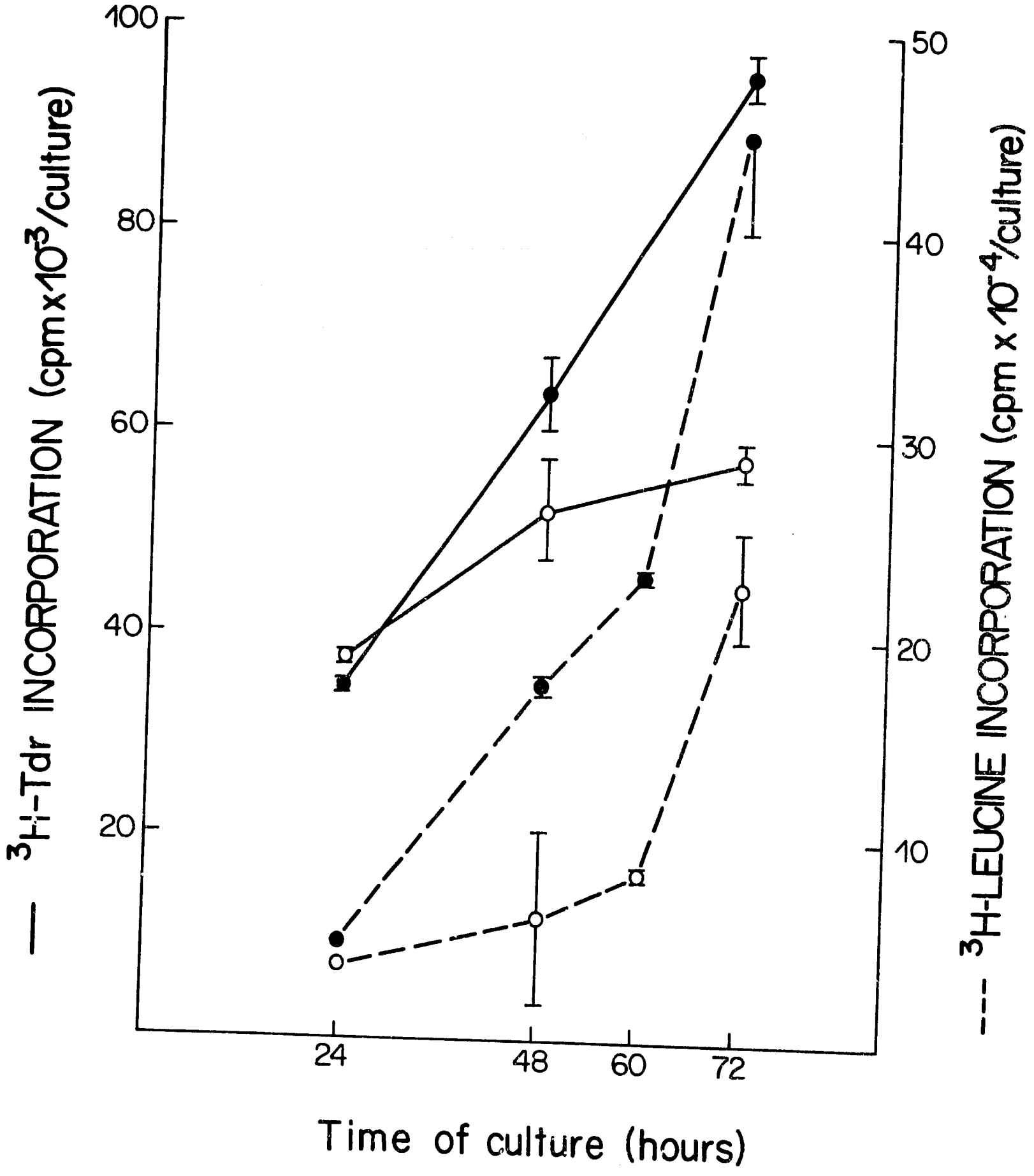
FIGURE LEGENDS

Fig. 1. Effect of LRSN on the growth of E. histolytica. To test the effect of LRSN on cultures of E. histolytica trophozoites, 4 ml of LRSN were added to 9 ml of medium containing 54,000 trophozoites in the exponential phase of growth (72 hr culture). The cultures were incubated at 37°C and the number of amebas determined. The data represent the mean ± standard error of 11 experiments. The p value was determined by Student's t test.

Fig. 2. Effect of LRSN on protein or DNA synthesis. The effect of LRSN on either protein or DNA synthesis was determined by measuring the incorporation of either [^{3}H]-leucine of [^{3}H]-TdR by E. histolytica trophozoites as described in Materials and Methods. The figure shows that LRSN inhibited the levels of protein and DNA synthesis after 48 hr of culture. The data represent the mean ± standard error of five experiemnts for [^{3}H]-leucine incorporation and of six experiments for [^{3}H]-TdR incorporation.


100
80
60
40
20
TROPHOZOITE NUMBER X 10^-4
24
48
72
TIME OF CULTURE (HOURS)

98


100
80
60
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50
40
30
20
10
24
48
60
72
— ^{3}H-Tdr INCORPORATION (cpm x 10^{-3}/culture)
--- ^{3}H-LEUCINE INCORPORATION (cpm x 10^{-4}/culture)
Time of culture (hours)