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MANAGEMENT FINAL REPORT

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DEVELOPMENT.

"ANTI-E.HISTOLYTICA MONOCLONAL IgA: ITS ROLE IN PROTECTION MECHANISMS".

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1. EXECUTIVE SUMMARY

The main objective of this project has been to investigate the role of the secretory immune response in amebiasis, a parasitic disease acquired through the oral route that causes infection without symptomatology in 90% percent of the infected individuals, however, 10% of them develop intestinal or extraintestinal invasive forms of the disease.

In search for the original objectives we produced monoclonal anti-*E.-histolytica* IgA antibodies, fusing Sp2 mouse myeloma cells and lymphoid cells from spleen or Peyer's patches from intragastrically immunized Balb/c mice. For the immunization procedure axenically cultured *E.histolytica* HIM1:IMSS were added with secretory immune response adjuvants (muramyl dipeptide or cholera toxin). IgA hybridomaspecific for *E.histolytica* were selected, cloned and characterized.

On the other hand, biological characterization was performed. For this purpose, two specific clones were selected: F1P1D4, F1P1D5 which recognized a doublet > 200 Kd and a 60 and 30 Kd protein band of ameba antigen respectively; the recognized epitopes were localized in the cytoplasmic membrane of trophozoites. Since adhesion of *E.histolytica* trophozoites to target cells in the host intestine is the first of three consecutive steps (adhesion, cytolytic effect and phagocytosis) involved in the invasion of colonic tissues, one of the main effects of an effective secretory antibody response must be the inhibition of the first most important step in colonizing the intestinal mucosa (adhesion). To test the participation of IgA in the inhibition of trophozoite adherence to target cells we tested two approaches: inhibition of *E.histolytica* adherence to HT-29 human colonic adenocarcinoma cells or to MDCK dog epithelial kidney cells. In situ experiments were also performed using the intestinal mucosa from mouse or gerbils (*Meriones unguiculatus*) as targets since both species seem to differ in their susceptibility to intestinal infection. In both models (*in vitro*

and *in situ*) monoclonal IgA inhibited the adhesion process. However, studies to test the participation of IgA anti-*E.histolytica* in the establishment of extraintestinal lesions (amebic liver abscess) in hamsters or gerbils did not modify the establishment nor the development of the amebic liver abscess.

Another biological property of IgA in the secretory immune response, observed in other parasitic, bacterial and viral intestinal diseases, is the antibody dependent cell cytotoxicity (ADCC) in which IgA is involved. This phenomenon was explored using spleen, mesenteric nodules or Peyer's patches lymphoid cells, however, the presence of anti-*E.histolytica* IgA monoclonal antibodies in the *in vitro* lymphoid cell-trophozoite interaction, both from immune and non-immune mice, was not determinant for the trophozoite cytolysis in the *in vitro* ADCC model.

These observations in our opinion are in agreement with the function of IgA as a first line of defense in the mucosal immune system.

2. RESEARCH OBJETIVES

One of the aspects that was escarcely investigated until the 90's is the role of the secretory immune response in the *E.histolytica* infection. Since amebiasis is acquired through the oral route it was important to explore an immune mechanism that can interfere with the first stages of the host-parasite relationship. The role of IgA in these first stages has been documented in other gastrointestinal infection diseases (1,2,3) and more recently, in amebiasis (4). This project adds new knowlege to the field. As planned we produced IgA monoclonal antibodies that were useful to explore participation of IgA in the first step of the interaction between target cell and trophozoite.

This is particularly important while the scientific community working in amebiasis is making efforts towards the development of a vaccine to prevent infection (5,6,7). Even though extraintestinal amebiasis (amebic liver abscess for example) is the most frequent

complication of the invasive intestinal amebic infection, it is important to learn first about mechanisms of the host-parasite relationship susceptible of being blocked through immunological stimulation at the intestinal level. At present, the stimulation of effective immune responses at mucosal surfaces are a central point in the design of vaccines for a number of different infectious diseases for which the point of entry are mucosal surfaces (7,8,9,10,11).

On the other hand, two of the studied monoclonals recognized epitopes located three different antigenic fractions with similar molecular weight to other proteins described in the literature (12,13), some of which have adhesion properties to the trophozoite.

The development of this project allows the creation of an alternative research line on the host-parasite relationship in infected human individuals, in which IgA antigenic recognition patterns were analyzed (14,15) In this particular case, the described epitopes recognized by our IgA monoclonals are also recognized by serum IgA from infected individuals, however, we still lack the precise characterization of the antigenic fractions, to define if there is more than one protein with the same molecular weight, which would permit us to determine the protein where the epitopes recognized by our monoclonals are located.

In our opinion, the impact derived from this project, to improve public health in developing countries will come from the efforts directed towards the study of better animal models of amebic disease, which can be used to test various ways of inducing an effective secretory immune response at intestinal level to avoid colonization by pathogenic trophozoites.

Many experimental animal models for development of the intestinal disease have been tested, however, most of them only partially reproduce the anatomopathological events of intestinal lesions in human amebiasis (16,17,18). Due to the high difficulty involved in reproducing previously published disease models, we only obtained consistent results in the highly reproducible amebic liver abscess model.

Regarding the financial support for the present project, it must be said that other research lines have derived from this project, and some of them have been supported by the National Council for Science and Technology (CONACYT) in Mexico.

3. METHODS AND RESULTS.

IgA-anti *E.histolytica* monoclonal production.

In previous reports (19) we found that immunization of Balb/c mice with *E.histolytica* trophozoites using the oral route, induces a local and systemic anti-ameba antibody response.

Even through this is an efficient route for IgA antibody production, the obtained levels were not high enough to assure the production of IgA hybrids. For this reason two groups of Balb/c mice were intragastrically (IG) immunized with *E-histolytica* trophozoite membrane extract antigen (500 mg), in the presence of muramyl dipeptide (MDP) (100µg) administered in 3 doses on consecutive days, repeated 15 days later and then intraperitoneally (IP) inoculated (100 µg of antigen) 16 and 32 days after the last immunization (Table 1, 2).

The second group of mice was immunized IP with 2.5×10^6 trophozoites and 20 µg of cholera toxin (CT) administered on two occasions with a 15 day interval; 45 days later, mice were boosted IG with 5×10^6 trophozoites plus 20 µg of CT. Mice that responded successfully were chosen for hybridization. MDP and CT were used as adjuvants for IgA antibody synthesis (Table 1,2).

Hybridization was performed by fusing Sp2/0-Ag 14 mouse myeloma cells with a pool of lymphoid cells from Peyer's patches (PP) or spleen cells (ratio 5:1). Fusion and clonation of hybrids were performed as previously described (20). The hybridization with PP cells from MDP immunized mice, produced five IgA-and one IgG-secreting hybridoma. Fusion with PP cells from mice immunized with CT produced seven IgA and two IgG parental hybrids. Unfortunately, fusion with spleen lymphoid cells did not produce anti-ameba secreting hybrids. Results of the hybrid production are shown in Table 23 After

clonation we selected specific hybrids (Table 4) to conduct the biological studies and the non-specific clones (anti-amoebic monoclonal antibody producers) were kept frozen for special experiments.

To identify the structures of trophozoites recognized by selected IgA monoclonal antibodies, indirect immunofluorescence studies were performed with antibodies produced by F1P1D4 and F1P1D5 clones, which are specific clones with IgA isotype. For this purpose, 48 h cultured axenic *E.histolytica* HM1-IMSS trophozoites (21) (2×10^5) were mixed with 250 μ l of monoclonal dilution, incubated (4°C, 30 min) and washed (200g, 15 min/4°C) with 0.02 M phosphate buffer (PBS) pH 7.4. Anti-mouse IgA α -chain specific antibody conjugated fluoresceine (1:40) was used to reveal the antigen-antibody reaction. The pellet was washed as before and then fixed with a 4% formaldehyde solution. Some of the assays performed allowed cap formation (Fig. 1).

Western blot was performed as originally described (21,22). Monoclonal antibody F1P1D4 recognized a doublet of approximately 200 KDa, F1P1D5 monoclonal IgA recognized two molecules, of 60KDa and 30KDa (Fig. 2). Affinity chromatographic purification of F1P1D5 monoclonal antibody (sepharose-anti-mouse IgA) under non-reducing and reducing conditions suggest that this monoclonal is secreted in dimeric form (Fig.3).

Antibodies produced by clones F1P1D4 and F1P1D5 are clearly directed to epitopes associated to cellular membrane molecules as shown by the redistribution of these epitopes and cap formation (Fig.1). Results obtained in the Western blot assays indicate that the IgA monoclonal antibodies of the studied clones recognize epitopes related to different antigenic fractions (Fig.2).

***Entamoeba histolytica* ADHERENCE: INHIBITION BY IgA MONOCLONAL ANTIBODIES.**

This part of the project was performed in two different systems. For the *in vitro* assay we used *E.histolytica* HM1:IMSS trophozoites and as target cells the dog MDCK cell

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line and the adenocarcinoma human cell line HT-29. In both cases, target cells and trophozoites formed rosettes due to the multiple adhesion molecules on the surface of both types of cells (target and trophozoites). This approach is frequently used to test the characteristics of the *E.histolytica* adherence process, which is the first step to occur leading to tissue damage.

Based on this fact we intended to inhibit the adhesion of trophozoites to the target cells with the IgA monoclonal antibodies (Fig. 4).

We used two anti-*E.histolytica* specific monoclonal IgA antibodies and one anti-ameba monoclonal IgA antibody, we also included two non-related IgA monoclonals (MOPC-315 and TPC15) as controls. Results show that inhibition of adherence due to the presence of anti-*E.histolytica* antibodies is greater (Table 5) than inhibition by non-specific anti-ameba monoclonal antibodies (Table 56). The analysis of these results suggest that non-specific mechanisms also participate in the inhibition process since some effect was observed with a non-related monoclonal IgA. This is non surprising, IgA is a molecule with considerable carbohydrate contents, it may easily contribute to the interaction with trophozoite surface molecules however, specific ligands seem to be the major targets for the inhibition of adherence between trophozoites and the epithelial cells.

Once the *in vitro* model was tested, we examined the inhibition capacity of the same IgA-anti-*E.histolytica* monoclonal antibodies in the *in situ* system. For this purpose, gerbil and mouse intestinal mucosa was selected as target tissue since these animals show differences in genetic susceptibility to *E.histolytica* infection. In this case *E.histolytica* HMI:IMSS trophozoites were radiolabeled (³H-thymidine with 20 Ci/mMol of specific activity), and incubated with sections of intestinal mucosa in the presence and absence of monoclonal antibodies. Results are shown in Table 7. Again, anti-*E.histolytica* IgA antibodies inhibited the adherence of trophozoites to the intestinal mucosa. No important apparent differences were detected in the two animal species (23,24).

STUDY OF THE ROLE ANTI-*E.histolytica* IgA ANTIBODIES IN THE ESTABLISHMENT OF HEPATIC AMEBIASIS IN THE HAMSTER MODEL.

The hamster model of amebic infection is among the most reproducible animal models of infection by *E.histolytica*. In this case we used hamsters weighing 90g, previously anesthetized (Ketalar) for surgery, and inoculated them with 2×10^5 trophozoites in 0.1 ml of TY1-S-33 culture medium into the Portal vein (18).

Results are shown in Table 7. The analysis of these results suggests that the establishment of previously opsonized trophozoites with anti-*E.histolytica* monoclonal IgA is not modified, since the inoculated hamsters developed amebic liver abscess with the same characteristics in size and in anatomopathology of hepatic damage as in control animals inoculated with un-treated trophozoites.

The same experiment was performed with trophozoites suspended in 90 µg of monoclonal solution in TY1-S-33 culture medium. Once again the development of amebic liver abscess was not modified (Table 8). These results suggest that the presence of circulating IgA is not a limiting factor for the establishment of *E.histolytica* trophozoites and for the development of extraintestinal amebic lesions.

IgA ANTIBODY DEPENDENT CELL MEDIATED CYTOTOXICITY (ADCC) AGAINST *Entamoeba histolytica*.

ADCC is recognized as one of the immune mechanisms involved in the elimination of parasites, bacteria or viruses. However, it has not been extensively studied in the intestinal infections by protozoa. One of the effects attributable to secretory IgA is its participation in the ADCC mechanisms in the intestine (25,26).

In the present project, the effect of anti-*E.histolytica* monoclonal antibodies in the ADCC response against trophozoites was studied. For this purpose, axenically cultured *E.histolytica* HM1: IMSS trophozoites (27) were incubated with lymphoid cells from spleen,

Peyer's patches or mesenteric nodules from immunized and non-immunized mice in the presence or absence of anti-*E.histolytica* monoclonal antibodies and the proper controls.

Results indicate that the anti-*E.histolytica* monoclonal IgA tested, seems not to play an important role in the ADCC phenomenon, however, the immune intestinal fluid from orally immunized mice and the lymphoid cells from PP and MN showed a cytotoxic effect on *E.histolytica* trophozoites. On the other hand, serum anti-ameba antibodies in conjunction with spleen lymphoid cells from immunized mice also showed a clear but lower cytotoxic effect. Results are shown in Tables 9,10 AND 11.

The failure to obtain cytotoxic effects on *E.histolytica* trophozoites with anti-*E.histolytica* monoclonal IgA does not discard the possibility of antiamebic secretory IgA participation in ADCC at intestinal level, since the intestinal fluid from orally immunized mice, tested for the presence of high titers of anti-*E.histolytica* IgA antibodies (data not shown), in conjunction with lymphoid cells from orally immunized mice, can generate a cytotoxic effect of more than 50% trophozoite mortality in the tested experimental system . This may reasonably support the idea of a possible role of IgA in other immune mechanisms for *E.histolytica* elimination which could operate at intestinal level. However, much has to be learned in this respect, specially concerning the possible relevance that these experiments could have *in vivo*.

In summary, the role of anti-*E.histolytica* IgA in the host-parasite relationship seems to be a first line of defense in the intestine, probably interfering with the first important step (adhesion) for succesful amebic colonization and tissue invasion of the large intestine.

4. IMPACT RELEVANCE AND TECHNOLOGY TRANSFER.

Concerning this particular point, at the time of approval of our original proposal there was no strict indication for obligatory collaboration with another developing country, so we cannot inform about this at present.

However, the development of this scientific project has allowed us first, to acquire the necessary expertise in the elaborated discipline of hybridization of immune lymphoid cells from gut-associated lymphoid tissue to produce monoclonal IgA. We have trained several laboratory members and students in this technique, and we will very soon produce other monoclonals which will contribute to the knoweldge on the local immune response in amebiasis (see Trained Individuals, part 5).

On the other hand, this project is still the central research line in our laboratory so all the acquired equipment is highly important for the infrastructure of the Infectology Laboratory at the Experimental Medicine Department in the Faculty of Medicine of the National University of Mexico (UNAM). This allow us, not only to continue our work, but also to receive students and train them in this area. (Master in Science degree, or PhD. levels).

5. LIST OF SCIENTIFIC MEETINGS ATTENDED DURING THE PROJECT.

-Ximénez Cecilia, Morán Patricia, Ramos Fernando, Leyva Oscar y Melendro EI. Specific anti *E.histolytica* IgA Monoclonal antibodies. 7th International Congress of Mucosal Immunology, August 16 to 20, Prague, Czechoslovakia, 1992.

- Ramos Fernando, Leyva Oscar, Rico Guadalupe, Morán Patricia. Melendro EI, and Ximénez Cecilia. *E.histolytica* Adherence: Inhibition by IgA monoclonal antibodies. 7th International Congress of Mucosal Immunology August 16 to 20, Prague, Czechoslovakia, 1992.

- Ximénez Cecilia, Morán Patricia, Ramos Fernando, Leyva Oscar y Melendro EI. Specific anti-*E.histolytica* IgA monoclonal antibodies. II Encuentro Hispanoamericano de Parasitología, March, 1992 Mexico, D.F.

- O.Leyva, G.Rico, F. Ramos, P.Morán, EI Melendro, and C. Ximénez. Inhibition of Adhesion Process Mediated by Anti-*Entamoeba histolytica* Specific Monoclonal IgA Antibodies. XII Seminar on Amebiasis, November 1992 Mexico D.F.
- O. Leyva, G. Rico, F. Ramos, P. Morán, EI Melendro, and C. Ximénez *E.histolytica* adherence: Inhibition by IgA monoclonal antibodies VII PAABS International Congress of Biochemistry, Ixtapa, Zih,. October 1992.
- Ramos F., Leyva O. Rico G. Morán P. Melendro EI. and Ximénez C. *E.histolytica* Adherence: Inhibition by IgA Monoclonal antibodies. XIX Congress of The Mexican Biochemical Society, Ixtapa, México, September 1992.
- Alvarado A, Morán P, Drago E, González E. Ramos F, Melendro EI, Ximénez C. IgA antibody dependent cell-mediated cytotoxicity against *E.histolytica* trophozoites. National Congress for PhD students March, 1995. Mexico D.F.

6. PROJECT PRODUCTIVITY.

Along the development of this project we reached all proposed objectives. We trained students and technicians in cell-line cultures, culture of ameba trophozoites under axenic conditions, hybridoma production, separation techniques for lymphoid cells from gut-associated lymphoid tissue, immunoenzymatic techniques for antigen or antibody characterization, macromolecule purification techniques, manipulation techniques for oral, intragastric, and intraperitoneal immunization of experimental animals, production of amebic liver abscess in hamsters and gerbils through intraportal inoculation of trophozoites , and others.

On the other hand, we determined that, at least in the experimental models of invasive amebic disease (ALA), the presence of IgA in serum. seems not to be a limiting step for the establishment of ameba trophozoites in the liver. However, all results obtained point to the participation of IgA in the first stages of the host-parasite relationship where

adherence of trophozoites to the intestinal mucosa determines the following events, namely cytolysis and invasion of the deep intestinal tissues or of extraintestinal sites such as liver, brain, lungs, and others..

PAPERS IN SPECIALIZED JOURNALS.

-Villarreal V. Melendro EI., Ramos F, and Ximénez C. Local and Systemic antibody response in Balb/c mice immunized with *E.histolytica* trophozoites. Archives of Medical Research, 23:69-72, 1992.

-Leyva O, Rico G, Ramos F, Morán P, Melendro EI. and Ximénez C. Inhibition of THE adhesion process mediated by anti-*Entamoeba histolytica* specific monoclonal IgA antibodies. Archives of Medical Research 23: 227-229, 1992.

-Leyva O, Rico G, Ramos F, Morán P, Melendro EI, and Ximénez C. *E.histolytica* adherence: Inhibition by IgA monoclonal antibodies In: Recent advances in Mucosal Immunology. Part A. McGhee J, Mestuby J, Tlaskalova H, Sterzl J Ed Plenum Presblishing Corporation. pp 681-683, 1995.

-Ximénez C. Morán P. Ramos F., Leyva O. and Melendro EI. Specific anti-*E.histolytica* IgA monoclonal antibodies In: Recent Advances in Mucosal Immunology. McGhee J, Mestecky J, Tlaskalova, Sterzl J. Ed. Plenum. Publishing Co., Part. A. Pg. 681-683, 1995.

-Gómez A, Leyva O, Martínez MC, Garduño G, Ramos F, Morán P, Melendro EI, Muñoz O, Ximénez C. Proliferative study of *Entamoeba dispar* infection in a cohort of pregnant women and their infants: epidemiological findings and their relationship to systemic immune responses. Am. J. Trop. Med. Hyg. accepted for publication.

Manuscripts in preparation.

- Alvarado A, Morán P, Drago E, González R, Ramos F, Melendro EI, Ximénez C. IgA Antibody dependent cell mediated cytotoxicity against *E. histolytica* trophozoites. (sent for publication) Arch. Med.
- Ramos, F, González E, Pérez-Ramayo R, Alvarado A, Morán P, Melendro EI, Ximénez C. Intestinal absorption of *E. dispar* antigen: Alternative paths of systemic immunization.(in preparation).

Graduated students under the project.

- Guvia Sosa 1992. Chemistry Sciences
- Enrique González Rivas 1993, Biology
- Patricia Morán Silva MD 1994, Master in Biomedical Sciences
- Oscar Leyva López 1995, Master Biological Sciences.
- Anabell Alvarado Navarro, Master in Biomedical Sciences (Preparing her manuscript).

Trained personel from other laboratories and Universities.

- Two technicians from the Dermatology Center, University of Guadalajara, Jalisco,. Mexico (production of monoclonal antibodies and hybridization techniques for IgA monoclonal production).
- One MSc. student from the University of Mérida, Venezuela. (Isolation of lymphoid cells from gut-associated lymphoid tissue GALT).
- One MSc student form the Autonomous Metropolitan University (UAM), Mexico City Trained in Characterization of *Giardia lamblia* surface molecules associated with the cellular immune response in the experimental infection of gerbils. *In vitro* stimulation of lymphoid cells from Peyer's patches, mesenteric nodules and spleen with different antigenic fractions of *G. lamblia*

7. FUTURE WORK.

Three different research lines have derived from the original project.

a) One of them, which will support our original hypothesis about the participation of IgA in the limitation of the intestinal mucosa colonization by pathogenic *E.histolytica* trophozoites, is the epidemiological follow up study of cohorts of infected individuals, in which we are evaluating the secretory response in cohorts of cyst passers, intestinal amebic patients (symptomatic), patients with amebic liver abscess and the proper cohorts of controls (infected with other parasites, patients with acute diarrhea due to non-parasitic microorganisms, and healthy controls).

The secretory immune response will be evaluated through the detection of secretory antibodies in feces and saliva and we will then characterize the specificity of these antibodies through Western blot analysis of reactivity in human samples against pathogenic *E.histolytica* HM1: IMSS antigen.

b) This project is presently in process and it has led us to a simultaneous study of the pathogenic characteristics of *E.histolytica* strains isolated from feces samples of individuals included in the different cohorts. The strategies employed for this are zymodeme characterization and the amplification of DNA sequences specific for pathogenic or non-pathogenic strains through Riboprinting technique (27,28,29,30) This part is also in progress.

These two activities form a multidisciplinary project with the participation of: Internal Medicine Physicians, Epidemiologists, Parasitologists, Immunologist and Molecular Biologists. We have obtained interesting results, some of which will be published soon.

c) The third and most important alternative research line derived from this project, is the molecular characterization of the proteins recognized by our monoclonals, specifically those proteins also recognized by the serum IgA of infected individuals. This part of the

project has begun with the collaboration of other research groups working in Molecular Biology of Parasitic Diseases and it will allow us to widen and enrich our research field greatly .

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ABSTRACT OF ACHIEVEMENTS: PROJECT No. 936.5542.01-523-9.130

TITLE: "ANTI-*E.HISTOLYTICA* MONOCLONAL IgA ITS ROLE IN PROTECTION MECHANISMS.

TECHNICAL ACHIEVEMENTS:

- Production of IgA monoclonal antibodies againsts *Entamoeba histolytica*.
- Biochemical and Biological characterization of IgA anti *E.histolytica* monoclonal antibodies.

ACADEMICAL ACHIEVEMENTS:

a) Attendance to academic meetings where advancements of research were shown.

- IX National Congress of Immunology. 30 Oct, 2nd Nov. 1991. (two papers)
- 7th International Congress of Mucosal Immunology, Prague August 1992. (two papers, one symposium)
- VII PAABS Congress. International Congress of Biochemistry, October 1992 Ixtapa (Mex). (two papers)
- International conference on amebiasis. Nov. 1992 Mexico City (two papers)
- National Congress for PhD students 1995 Mexico City.

TABLA 1

IMMUNIZATION SCHEDULE AND INTESTINAL ANTI-*E.histolytica* ANTIBODIES

GROUP	SCHEDULE	ANTIBODY CLASS	
		IgG	IgA
1	2IG-MDP + 2IP	0.370	0.435
2	2IP + 1 IG-TC	0.705	1.150

ELISA of intestinal fluid used for fusion. Values correspond to the mean O.D. at 490 nm minus control values. 3 Ig-MDP doses were administered on consecutive days.

TABLA 2

IMMUNIZATION SCHEDULE AND SERUM ANTI- *E.histolytica* ANTIBODIES

GROUP	SCHEDULE	ANTIBODY CLASS	
		IgG	IgA
1	2IG-MDP + 2IP	0.850	1.150
2	2IP + 1 IG-TC	0.750	1.500

ELISA of sera used for fusion. Values correspond to the mean O.D. at 490 nm minus control values

3 Ig-MDP doses were administered on consecutive days.

et

TABLA 3

ANTI-AMEBA HYBRIDOMA PRODUCTION

IMMUNIZATION SCHEDULE	CELL SOURCE	WELLS EXAMINED	ANTI-AMEBA ANTIBODY	
			IgG	IgA
2IG-MDP + 2IP	PP	118	1	5
	SPLEEN	98	0	0
2IP + IG-TC	PP	130	2	7
	SPLEEN	103	0	0

Non IgM producing hybridomas were obtained

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TABLA 4

SPECIFICITY OF ANTI- *E.histolytica* MONOCLONAL ANTIBODIES

PARENTAL CLONES	ISOTYPE	<i>E.histolytica</i>		<i>E.invadens E.histolytica</i>	
		HM1:IMSS	HK9:NIH	<i>LIKE (LAREDO)</i>	
F1P1D4	IgA	0.86	0.93	0.09	0.08
F1P1D5	IgA	0.42	0.42	0.08	0.06
F1P2D5	IgA	0.48	0.60	0.10	0.09
F1P1G10	Iga	0.42	0.37	0.09	0.09
F1G1F2	IgG	0.35	0.36	0.04	0.06

ELISA of culture supernatants. Values correspond to the mean O.D. at 490 nm minus control values.

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TABLE 5
INHIBITION OF *E. histolytica* ADHERENCE TO MDCK OR HT-29 CELL LINE^a

CELL LINE	ANTIBODY	ADHERENCE ^b (%)	INHIBITION ADHERENCE (%)
MDCK	-	80	-
	SP2/O-Ag14	76	16
	F1P1D4	31	62
	F1P1D5	13	85
	HYPERIMMUNE SERUM	0	100
HT-29	-	90	-
	SP2/O-AG14	87	13
	F1P1D4	3	97
	F1P1D5	33	64
	HYPERIMMUNE SERUM	36	60

a. Three different experiments were performed using a cell/trophozoites ratio of 20/1. Anti-*E. histolytica* IgA monoclonal antibodies (F1P1D4, F1P1D5) were used to inhibit the adhesion of both epithelial cell lines to trophozoites.

b. Values represent % of rosette formation (one-trophozoite with more than 3 epithelial cells attached)

TABLE 6
INHIBITION OF *E. histolytica* ADHERENCE TO MDCK OR HT-29 CELL LINE^a

CELL LINE	ANTIBODY	ADHERENCE ^b (%)	INHIBITION ADHERENCE (%)
MDCK	-	85	-
	SP2/0-Ag14	87	0
	H10B6F6	39	53
	MOPC 315	85	0
	TPC 15	80	5
	HIPERIMMUNE SERUM	0	100
	HT-29	-	88
SP2/0-aG14		83	6
H10B6F6		46	47
MOPC 315		86	3
TPC 15		67	24
HYPERIMMUNE SERUM		36	60

a. The three different experiments were made in the presence of non specific anti-amebic IgA monoclonal (H10B6F6) or non-related IgA mice monoclonal antibodies (MOPC315, TPC15)

b. Values represent % of rosette formation (one-trophozoite with more than 3 epithelial cells attached).

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TABLE 7

INHIBITION OF *E. histolytica* ADHERENCE TO FIXED MICE OR GERBILS COLONIC MUCOSA

		INHIBITION OF ADHERENCE (%)			
		A N T I B O D Y			
ANIMAL	ASCITIC FLUID ^a	F1P1D4 ^b	F1P1D5 ^b	HYPERIMMUNE ^c SERUM	
BALB/c					
MICE	-	40	49	74	
GERBILS	-	41	67	48	

a. Ascitic fluid from Balb c mice inoculated with 2.5×10^5 Sp2/0-Ag 14 myeloma cells, precipitated with a saturated ammonium sulphate solution (50% V-V) and dialyzed against PBS 0.02M pH 7.4. 90 μ g of protein in 500 μ l PBS were used in each experiment.

b. Affinity purified anti-*E. histolytica* monoclonal IgA antibodies were adjusted to 90 μ g of PBS.

c. 500 μ l of serum from Balb c mice inoculated with *E. histolytica* HM1:IMSS

TABLE 8

EFFECT OF ANTI-AMEBA IgA MONOCLONAL ANTIBODY IN AMEBIC LIVER ABSCESS

Groups of inoculated Hamsters ^a	<i>E. histolytica</i> HM1:IMSS trophozoites ^b				
	Non treated	TPC 15 ^c		F1P1D4 ^c	
		1	2	1	2
5	86	95	90	82	78 ^d

- a. Groups of 5 hamsters inoculated with a highly virulent HM1:IMSS strain (5×10^5 trophozoites) through Portal vein canalization
- b. Trophozoites were treated for opsonization with IgA monoclonal antibodies (1) or suspended in IgA monoclonal TY1-S-33 culture medium (2).
- c. TPC-15 is a commercial IgA monoclonal antibody (Sigma Chemical), F1P1D4 is IgA anti *E. histolytica* monoclonal antibody
- d. Values are expressed as % of hamsters with macroscopically evident amebic liver abscess.

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TABLE 9

CYTOTOXIC EFFECT OF SERUM OR INTESTINAL FLUID ON ^a *E.HISTOLYTICA* TROPHOZOITES

	Serum ^c		Intestinal Fluid ^c		Monoclonal IgA ^d Antibodies	
	Preimmune	Immune	Pre-immune	Immune	TPC15	F1P1D4
% of Death ^b Trophozoites	3.5	6.12	16.5	15	10.73	13.12 ^b

a. Controls included in all experiments

b. Values are the MEAN of three experiments

c. Preimmune sera and intestinal fluid were obtained from the same Balb/c mice before immunization with fixed (2.5% glutaraldehyde) *E. histolytica* HM1:IMSS trophozoites. Trophozoites were administered intragastrically (3 doses in consecutive days) every 15 days during 3 months.

d. TPC-15 is a non-related mice IgA monoclonal antibody and F1P1D4 is a mice anti-*E. histolytica* IgA ^a monoclonal antibody

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TABLE 11

AMEBICIDAL ACTIVITY OF LYMPHOID CELLS FROM *E.histolytica*
IMMUNIZED MICE IN THE PRESENCE OF SPECIFIC ANTIBODIES^a

ANTIBODIES ^b	LYMPHOID CELLS ^c		
	SPLEEN	PEYER'S PATCHES	MESENTERIC NODULES
	0	0	0
Preimmune serum	0	16.6	2
Immune serum	42	ND	27
F1P1D4	15	16	29
TPC15	3.8	4.1	0
Preimmune intestinal fluid	ND	16.6	8.3
Immune intestinal fluid	54.5	73.2	54

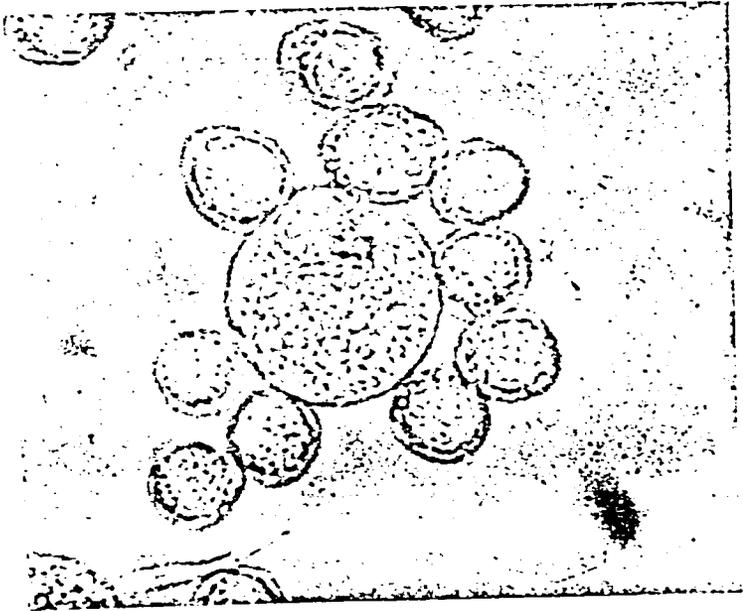
a. Trophozoite to cell ratio was 1:10

b. F1P1D4 is anti *E.histolytica* IgA monoclonal antibody, TPC15 is a commercial mice IgA antibody (Sigma Chemical Co) immune serum and intestinal fluid were obtained from orally immunized mice

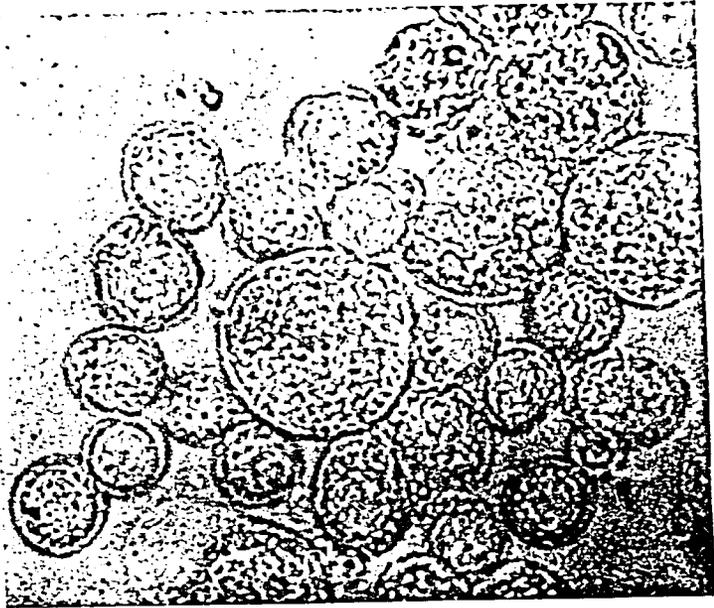
c. Values are the MEAN of three experiments expressed as % of death trophozoites.

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A



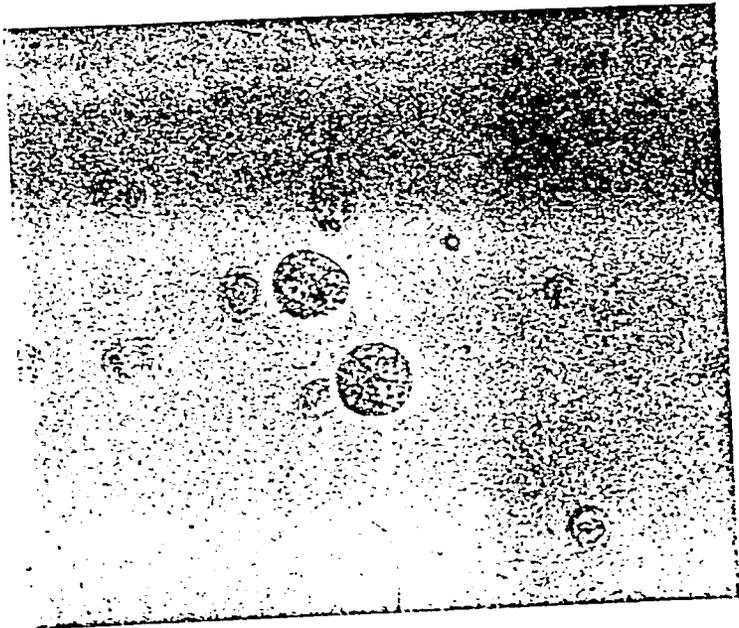
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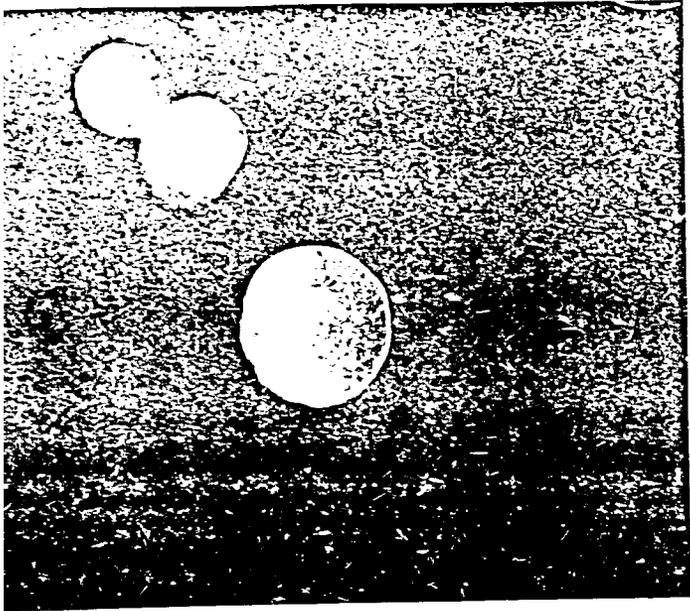
C



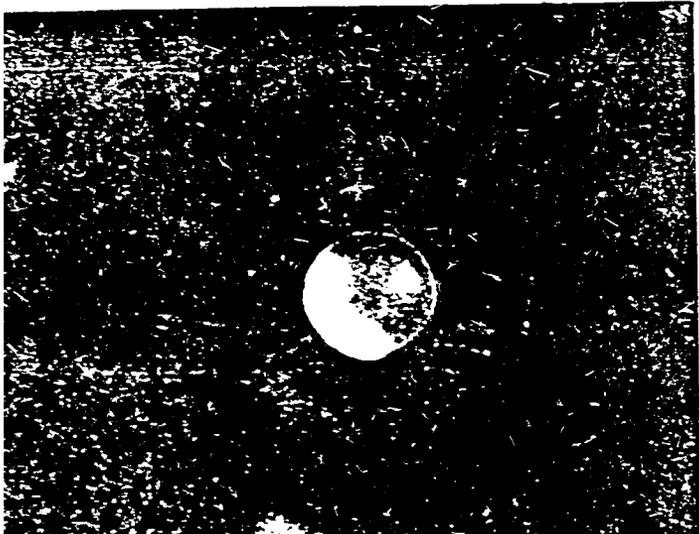
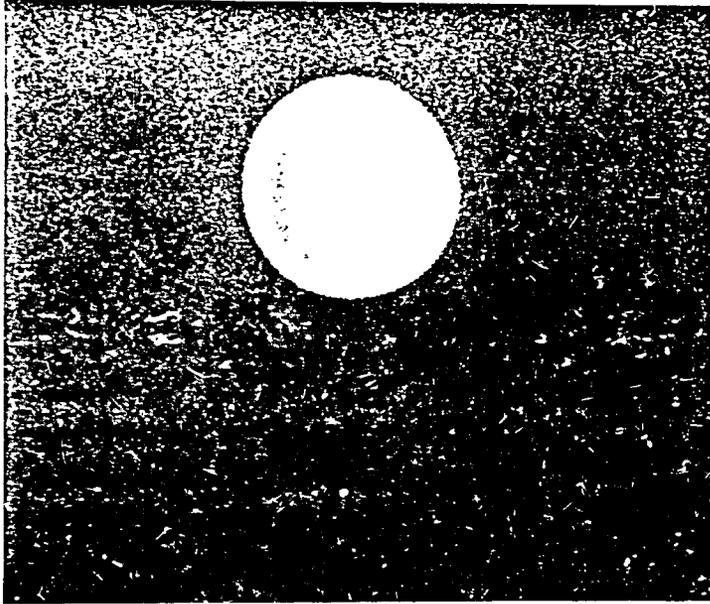
D



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B



D

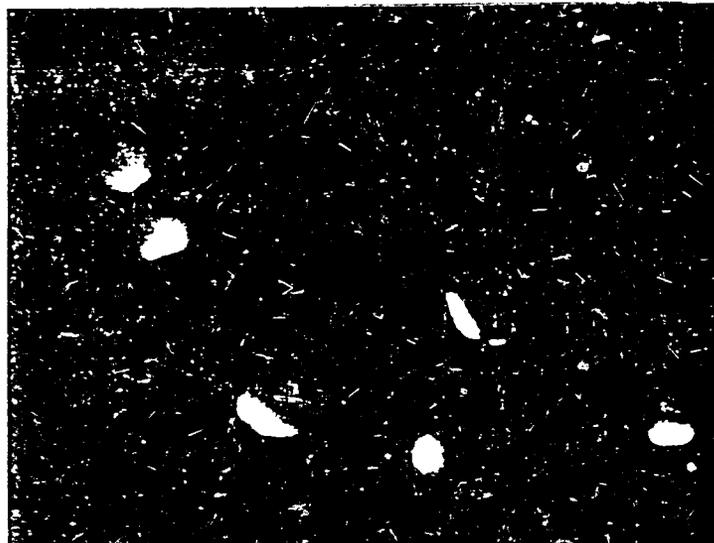


Fig.1. A) Rosette formation with E.histolytica HM1:IMSS trophozoites and HT-29 cells; B) in the presence of Sp2/O-Ag14 ascitis fluid. Inhibition of rosette formation with F1P1D4 (C) and F1P1D5 (D) anti E.histolytica monoclonal IgA.

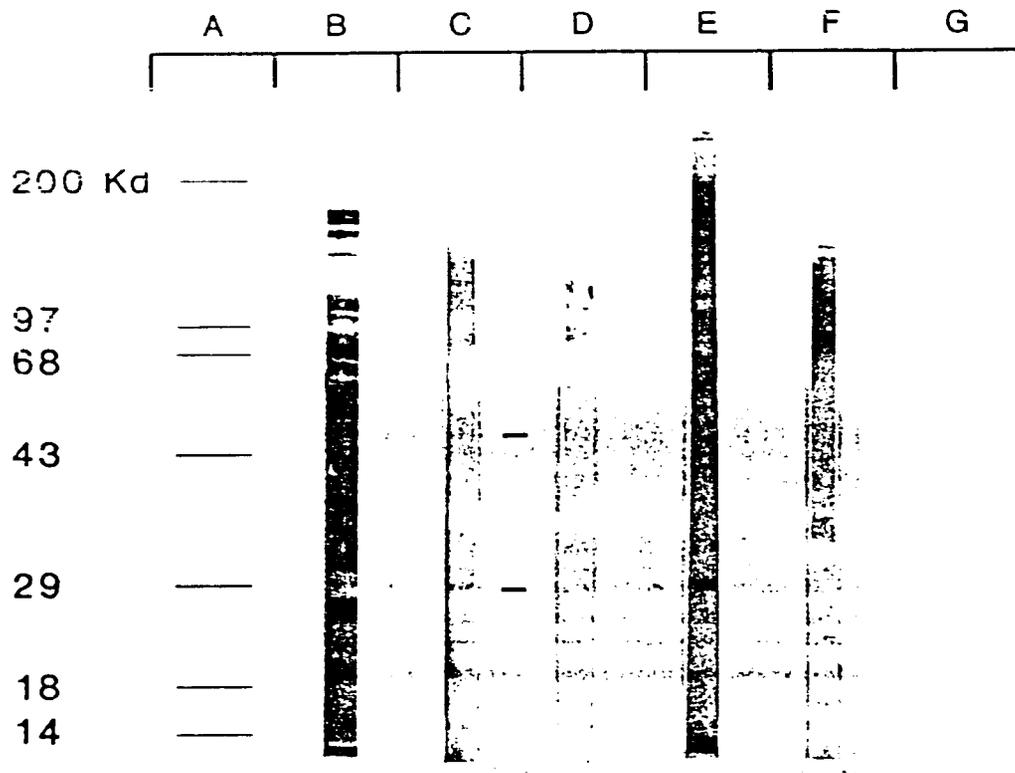


Fig.2. Western blot of *E.histolytica* HM1:IMSS membrane antigen revealed with specific monoclonal IgA antibodies; A) molecular weight (kD) ; B) *E.histolytica* antigen; C) F1P1D5 monoclonal antibody; D) F1P1D4 monoclonal antibody; E) hyperimmune serum; F) control with Sp2/0-Ag14 ascitis G) anti-mouse IgA peroxidase conjugated antibody

h/c

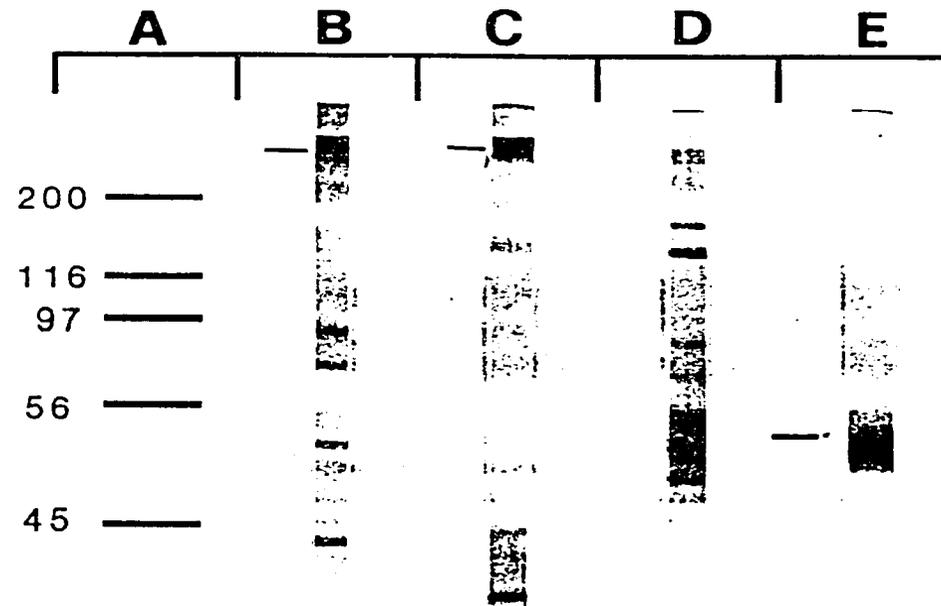


Fig.3. Electrophoresis of purified anti E.histolytica Monoclonal antibody in SDS-polyacrylamide gel; A) molecular weight (kD); B) F1P1D5 monoclonal IgA under non-reducing conditions; C) TPC15 mouse monoclonal IgA under non-reducing conditions; D) F1P1D5 under reducing conditions; E) TPC15 under reducing conditions.

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Fig.1. Immunofluorescence of E. histolytica HM1:IMSS trophozoites with monoclonal IgA antibody from clones F1P1D4 (A), F1P1D5 (B); capping of surface determinants in presence of monoclonal antibodies F1P1D4 (C) and F1P1D5 (D).

Inhibition of Adhesion Process Mediated by Anti-*Entamoeba histolytica* Specific Monoclonal IgA Antibodies

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Abstract

Adhesion of trophozoites to target cells in the host intestine is the first of three consecutive steps (adherence, cytolytic effect and phagocytosis) involved in the invasion of colonic tissue by *E. histolytica*. To investigate the possible participation of the local secretory immune response in the interference with this early host-parasite relationship, we produced IgA monoclonal anti-*E. histolytica* antibodies to test their capacity for blocking the adhesion process *in vitro* (MDCK and HT-29 cell lines) and *in situ* (colonic

mucosa from Balb/c mice and gerbils). Results demonstrate that the monoclonal antibodies tested inhibited trophozoite adherence both *in vitro* and *in situ*. This suggests that the local antibody response may play an important role in protection against the invasive process in intestinal amebiasis.

KEY WORDS: *Entamoeba histolytica*, IgA monoclonal antibody, MDCK cell line, HT-29 cell line, Colonic mucosa, Trophozoite adherence.

Introduction

Even though the mechanisms by which trophozoites of *E. histolytica* produce tissue damage in the intestinal mucosa still remain unclear, three consecutive steps are involved in the invasion by these protozoa: adhesion of ameba to target cells, contact cytolytic effect and phagocytosis (1).

Adhesion of trophozoites to target cells and to the extracellular matrix seems to be a prerequisite for the cytolytic and phagocytosis processes (2,3). The secretory immune response in amebiasis has not been sufficiently explored; however, it is possible that secretory IgA exerts a protective role interfering with some of the mechanisms associated with amebic virulence. To explore the role of IgA in the inhibition of adherence of *E. histolytica* trophozoites to MDCK and HT-29 cells *in vitro*, and to cecal mucosa from Balb/c mice and gerbils (*Meriones unguiculatus*), specific monoclonal anti-*E. histolytica* IgA was produced and tested for its capacity to inhibit the adherence of trophozoites.

Materials and Methods

Anti-*E. histolytica* monoclonal IgA. IgA from clones F1P1D4 and F1P1D5 produced by hybridization of SP2/0 Ag 14 mouse myeloma cells and Peyer's patches lymphoid cells from immune Balb/c mice were used for the inhibition of adherence of *E. histolytica* HM1:IMSS trophozoites to epithelial cell (MDCK and HT-29) assays. Controls included SP2/0 Ag14 ascitic fluid and anti-amebic hyperimmune sera from Balb/c mice.

Epithelial cell cultures. MDCK cells were kindly donated by Dr. G. Ortega, maintained and cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA) added

with 800 UI of insulin. HT-29 human colonic adenocarcinoma cell line was donated by Dr. J. Torres and routinely cultured in DMEM. Both MDCK and HT-29 cells were cultured in the presence of 10% decomplemented fetal bovine serum (GIBCO BRL), 1% non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml amphotericin B. Cultures were incubated at 37°C in a 95% air/5% CO₂ mixture.

Cultivation and radiolabeling of *E. histolytica* trophozoites. Asexually cultured *E. histolytica* trophozoites strain HM1:IMSS were cultured in TYI-S-33 medium (4) at 36.8°C and harvested after 48 h. Trophozoites (10⁶) were suspended in 4 ml of medium in 15 ml sterile tubes to which 20 µCi of ³H-thymidine (20 Ci/mMol) (New England Nuclear Corp., Boston, MA) were added. Tubes were incubated at 36.8°C for 24 h. After this, trophozoites were washed with fresh medium and suspended in it for 2 h to decrease the spontaneous release of radioactive label (5).

Inhibition of adherence of trophozoites to epithelial cells. Adherence of *E. histolytica* HM1:IMSS to MDCK or to HT-29 cells were studied by rosette formation as previously described (6). Trophozoites were harvested at the logarithmic growth phase and centrifuged (200 x g for 5 min at 4°C), the pellet was resuspended in DMEM at 10⁷ cells/ml. Specific monoclonal IgA were added (10 µg) and incubated (30 min, 4°C). Trophozoites were washed twice with fresh medium without serum. MDCK or HT-29 cells (2 x 10⁵) and previously treated trophozoites (10⁴) were suspended in 1 ml of supplemented medium, centrifuged (150 x g, 5 min) and incubated (4°C, 2 h). After incubation, 0.8 ml of the supernatant was removed and the tubes were gently stirred to homogenize the cell suspension. Percentage of ameba with three or more epithelial cells attached were calculated. Controls were included with trophozoites opsonized with Sp2/0 Ag-14, ascitic fluid or anti-ameba hyperimmune mouse serum (1:10).

Adherence of ³H-thymidine-labeled trophozoites to colonic mucosa. The cecum was removed from previously anesthetized (Ketalar) Balb/c mice and gerbils, and washed with 0.02 M phosphate buffered saline pH 7.4 (PBS) to eliminate fecal debris, cut in 3 mm sections and

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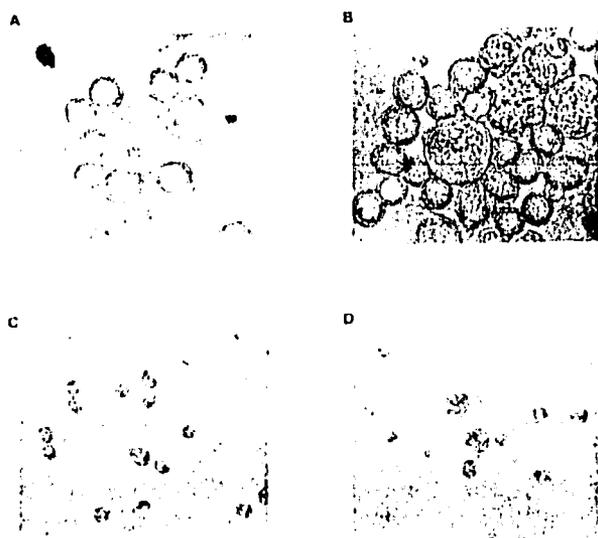


Figure 1. (A) Rosette formation with *E. histolytica* HM1:IMSS trophozoites and HT-29 cells; (B) in the presence of SP2/O Ag-14 ascitic fluid; (C) inhibition of rosette formation with F1P1D4 and (D) F1P1D5 anti-*E. histolytica* monoclonal IgA.

fixed in PBS containing 1% glutaraldehyde (24 h, 4°C). The tissue was placed in a plastic tube in TYI medium without serum. ³H-thymidine labeled trophozoites were added to the test tube and incubated (34°C, 30 min). To study the relevance of monoclonal anti-*E. histolytica* IgA in the inhibition of adherence of labeled trophozoites to colonic mucosa, trophozoites were incubated (37°C, 30 min) in the presence of the different monoclonal IgA, carefully resuspended, placed on the colonic tissue sections and then incubated at 34°C for 30 min. Tissue sections were removed from the test tube, washed in 1.5 ml of TYI medium without serum and placed in 7 ml scintillation fluid in 10 ml vials. Percent of adherent amebas was calculated as

Table 1. Inhibition of *E. histolytica* Adherence to MDCK or HT-29 Cell Line

Cell line	Antibody (%)	Adherence adherence (%)	Inhibition
MDCK		80	
	SP2/O-Ag14	76	16
	F1P1D4	31	62
	F1P1D5	13	85
	Hyperimmune serum	0	100
HT-29		90	
	SP2/O-Ag14	87	13
	F1P1D4	3	97
	F1P1D5	33	64
	Hyperimmune serum	36	60

Affinity purified anti-*E. histolytica* monoclonal IgA (F1P1D4 and F1P1D5) were used at a protein concentration of 100 µg/ml. Ascitic fluid from Balb/c mice inoculated with Sp2/O Ag-14 cells, previously precipitated with saturated (NH₄)₂SO₄ solution (50% v/v) was used at 100 µg/ml protein concentration. Anti-ameba hyperimmune serum was diluted 1:10; 100 µl vials were used in each case.

previously described (5).

Results

Inhibition of *E. histolytica*-trophozoite adherence to epithelial cells *in vitro*. The inhibition of adherence of *E. histolytica* HM1:IMSS trophozoites to MDCK or HT-29 cells *in vitro*, in the presence or absence of IgA-monoclonal antibodies, shows that these reagents were capable of efficiently inhibiting rosette formation (>60%) compared with control assays, where rosette formation (Figure 1) was almost complete. A comparison of inhibition mediated by IgA, with inhibition due to mouse anti-ameba hyperimmune sera, revealed no differences in the assays with HT-29 cells; however, with MDCK cells hyperimmune sera show an inhibitory capacity three-fold better than monoclonal IgA (Table 1).

Inhibition of *E. histolytica* trophozoites adherence to colonic mucosa. The adherence of trophozoites to the colonic mucosa was evaluated in two rodent species with different susceptibility to amebic infection. In the two species, adherence of axenic ³H-thymidine-labeled *E. histolytica* trophozoites to 3 mm sections of glutaraldehyde fixed colonic mucosa was 15.1 and 11%, respectively, after 30 min of incubation at 37°C. After this, we evaluated whether anti-*E. histolytica* IgA monoclonal antibodies inhibit amebic adherence in the *in situ* model. As shown in Table 2, monoclonal antibodies interfere with the process of adherence in about 50% compared with control assays, performed with Sp2/O Ag-14 ascitic fluid opsonized trophozoites; however, hyperimmune antiamebic serum showed no better inhibitory activity than monoclonal IgA antibodies.

Discussion

In the present study, experimental conditions were kept similar to those described in other target cells systems *in vitro* (6,7). The adhesion model with HT-29 cell line appears to be especially useful for adhesion studies of ameba to human cells. This cell line offers advantages over other epithelial cell lines since HT-29 cells are physiologically closer to normal target cells in amebiasis (8). The results of adherence inhibition with IgA monoclonal antibodies indicate that they recognized epitopes associated with molecules involved in the first step of tissue invasion. There is a correlation between the *in vitro* model and the adherence model of trophozoites to glutaraldehyde fixed colonic mucosa, both in Balb/c mice and gerbils. These two species show documented differences in susceptibility to *E. histolytica* intestinal infection (9,10). However, no differences in trophozoite adhesion to colonic mucosa of the two rodent species were observed in the *in situ* assay.

Table 2. Inhibition of *E. histolytica* Adherence to Mice or Gerbils Fixed Colonic Mucosa

Animal	Ascitic ^a Fluid	Inhibition of adherence (%)		
		Antibody		
		F1P1D4 ^b	F1P1D5 ^b	Hyperimmune serum ^c
Balb/c mice		40	49	74
Gerbils		41	67	48

^aAscitic fluid treated as indicated in Table 1 adjusted to a protein concentration of 90 µg/ml.

^bAffinity purified anti-*E. histolytica* monoclonal IgA (F1P1D4, F1P1D5) (90 µg/ml).

^cAnti-ameba hyperimmune serum was used as indicated in Table 1.

Our results suggest that even though it is difficult to clarify the biological significance of the secretory immune response in amebiasis, there is a reasonable possibility that local immunity plays a critical role in maintaining the balance between intestinal and luminal amebiasis.

Acknowledgments

We are grateful to I. Pérez Montfort for revision of the manuscript.

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SPECIFIC ANTI-*E. HISTOLYTICA* IgA MONOCLONAL ANTIBODIES

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INTRODUCTION

An important central question in the immunology of the amoebic infection is the role of the humoral immune response in protection mechanisms either in the intestine or at the systemic level. Studies on the role of anti-*Entamoeba histolytica* antibodies *in vivo* are few, but some epidemiological evidence has been obtained on the protective role of the local humoral immune response.¹ To find out how anti-amoebic IgA may contribute to protective mechanisms, we produced specific IgA monoclonal antibodies against *E. histolytica*. The present study describes the production and preliminary characterization of IgA monoclonals.

RESULTS

Two groups of BALB/c mice were intragastrically (IG) immunized with *E. histolytica* trophozoite membrane extract antigen (500 µg), in the presence of muramyl dipeptide (MDP) (100 µg) administered in 3 doses on consecutive days, repeated 15 days later and then intraperitoneally (IP) inoculated (100 µg of antigen) 16 and 32 days after the last immunization. The second group of mice, were immunized IP with 2.5×10^6 trophozoites and 20 µg of cholera toxin-(CT) administered on two occasions with a 15 day interval; 45 days later, mice were boosted IG with 5×10^6 trophozoites plus 20 µg of CT. Mice that responded successfully were chosen for hybridization. MDP and CT were used as adjuvants for IgA antibody synthesis.

HYBRID PRODUCTION

Hybridization was performed by fusing Sp2/0 Ag 14 mouse myeloma cells with a pool of lymphoid cells from Peyer's patches (PP) or spleen cells (ratio 5:1). Fusion and cloning of hybrids were performed as previously described.² The hybridization with PP cells from MDP immunized mice produced five IgA and one IgG secreting hybridoma. Fusion with PP cells from mice immunized with CT produced seven IgA and two IgG parental hybrids. Unfortunately, fusion with spleen lymphoid cells did not produce anti-amoeba secreting hybrids.

IMMUNOFLUORESCENCE

Indirect immunofluorescence studies were made with antibodies produced by FIPID4 and FIPID5 clones, which are specific clones with IgA isotype. For this purpose, 48

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h. cultured axenic *T. histolytica* HMI IMSS trophozoites¹ (2×10^5) were mixed with 250 μ l of monoclonal dilution, incubated (4°C , 30 min) and washed (200g, 15 min/ 4°C) with 0.02 M phosphate buffer (PBS) pH 7.4. Anti-mouse IgA α chain specific antibody conjugated fluorescein (1:40) was used to reveal the antigen antibody reaction. The pellet was washed as before and then fixed with 4% formaldehyde solution. Some of the assays performed allowed cap formation (Fig. 1).

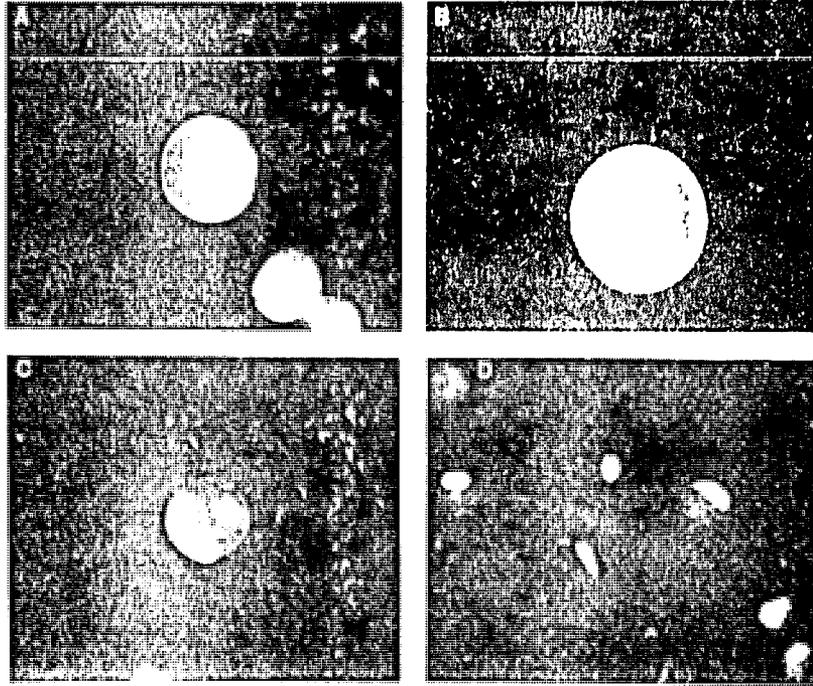


Figure 1. Immunofluorescence of *T. histolytica* HMI IMSS trophozoites with monoclonal IgA antibody from clones FIPID1(A), FIPID5(B); capping of surface determinant in presence of monoclonal antibodies FIPID1(C) and FIPID5(D).

WESTERN BLOT

Western blot was performed as originally described.¹ Monoclonal antibody FIPID4 recognized a doublet of approximately 200 kDa, FIPID5 monoclonal IgA recognized two molecules, of 50 kDa and 30 kDa (Fig. 2). Affinity chromatographic purification of FIPID5 monoclonal antibody (sepharose anti-mouse IgA) under non-reducing and reducing conditions suggest that this monoclonal is secreted in dimeric form (Fig. 3).

DISCUSSION

Oral administration of immunogens may induce a variety of responses, from an effective secretory immune response to the induction of states of tolerance, depending on the nature of the immunogen, the dose, the presence or absence of adjuvants of the secretory response and the strain of mice tested.^{2,9} In previous studies⁷ we have found that oral administration of *T. histolytica* trophozoites induces a relatively modest secretory response (IgA) after repeated immunization with trophozoites. However, the use of combined immunization methods (IG and IP route) together with MDP or CT significantly increases the levels of local and systemic IgA antibodies.

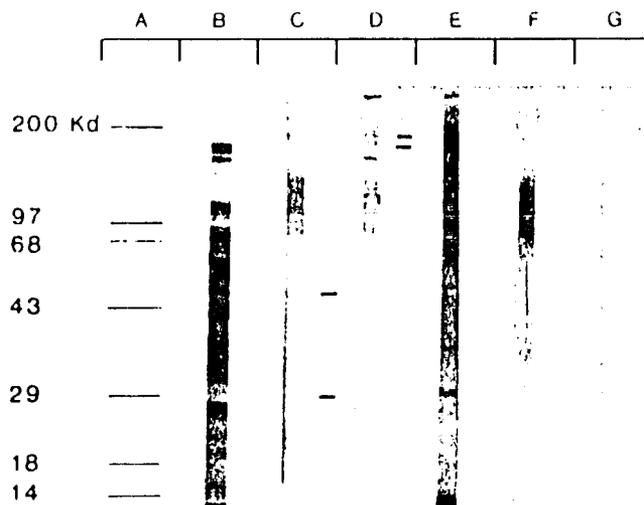


Figure 2. Western blot of *E. histolytica* HM1:IMSS membrane antigen revealed with specific monoclonal IgA antibodies; (A) molecular weight (kD); (B) *E. histolytica* antigen; (C) FIPID5 monoclonal antibody; (D) FIPID4 monoclonal antibody; (E) hyperimmune serum; (F) control with Sp2/0-Ag14 ascites (G) anti-mouse IgA peroxidase conjugated antibody.

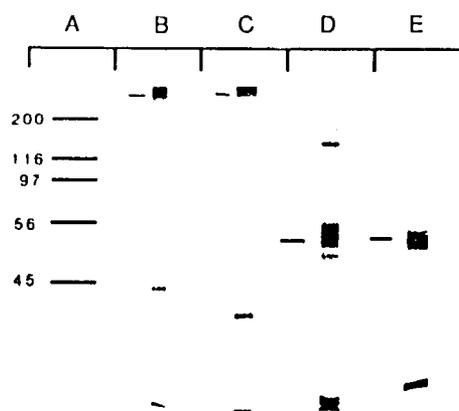


Figure 3. Electrophoresis of purified anti *E. histolytica* monoclonal antibody in SDS-polyacrylamide gel; (A) molecular weight (kD); (B) FIPID5 monoclonal IgA under non-reducing conditions; (C) TPC 15 mouse monoclonal IgA under non-reducing conditions; (D) FIPID5 under reducing conditions; (E) TPC15 under reducing conditions.

Antibodies produced by clones FIPID4 and FIPID5 are clearly directed to epitopes associated to cellular membrane molecules as shown by the redistribution of these epitopes and the capping formation (Fig. 1). Results obtained in the Western blot assays indicate that the IgA monoclonal antibodies of the studied clones recognize epitopes related to different antigenic fraction (Fig. 2).

The production of a homogeneous and highly specific reagent like a monoclonal antibody, in this case of the IgA class, may provide a useful tool for the study of the protective antiamebic immune mechanisms in the intestinal compartment.

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ACKNOWLEDGMENTS

This work was supported by the Agency for International Development, US-AID, Washington DC. USA grant No. 936-5542-02-5239, 130.

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ENTAMOEBEA HISTOLYTICA ADHERENCE: INHIBITION BY IgA MONOCLONAL ANTIBODIES

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INTRODUCTION

Adhesion of *Entamoeba histolytica* to target cells in the host intestine, is the first of three consecutive steps (adhesion, cytolytic effect and phagocytosis) involved in the invasion of colonic tissues.¹ The present study investigated the role of the local secretory immune response in the interference with this early host-parasite relationship. IgA monoclonal anti-*E. histolytica* antibodies were produced. One of the clones obtained (FIPID5) had been tested in its capacity to block the adhesion process *in vitro* with two different epithelial cells (MDCK and HT-29 cell lines), and *in situ* using colonic mucosa from BALB/c mice or gerbils (*Meriones unguiculatum*), which differ in susceptibility to *Entamoeba* experimental infection.

METHODS AND RESULTS

Inhibition of Trophozoite Adherence to Epithelial Cells *In Vitro*

Adherence of *E. histolytica* HM1: IMSS (10^4) to MDCK or HT-29 cells (2×10^5) was studied by rosette formation assays as previously described;² trophozoites were incubated with specific monoclonal IgA during 30 min at 4° C, then trophozoites were washed with D-MEM without fetal bovine serum, (GIBCO BRL, Grand Island, NY, USA). Trophozoites and MDCK or HT-29 cells were mixed (1 ml total volume) and after incubation (2 hrs 4° C) the supernatant (0.8 ml) was removed and the pellet was gently stirred. Percentage of amoeba with 3 or more cells attached was calculated. Results show that FIPID5 IgA monoclonal antibody inhibited rosette formation very efficiently (85 %) compared with control assays, where rosette formation was almost complete (Fig. 1). There are differences between inhibition adherence with MDCK compared to HT-29 human adenocarcinoma cell line (Table 1).

Inhibition of Adherence to Colonic Mucosa Assay

Two rodent species with different susceptibility to amoebic infection, were used to evaluate adherence of trophozoites to colonic mucosa. In both models adherence of axenically cultured [³H]-TdR labeled trophozoites,³ to 3 mm sections of fixed glutaraldehyde (2.5 %) colonic mucosa was 15 % after 30 min at 37° C. Subsequently we evaluated whether anti-*E. histolytica* IgA monoclonal antibody could inhibit amoebic adherence in the *in situ* model. As may be observed in Table 2, FIPID5 antibody, interferes with the adherence process between 50 % and 70 % compared with control assays in the absence of specific monoclonal antibody (non-opsonized trophozoites). However, hyperimmune mouse anti-amoebic serum shows no greater inhibitory activity.

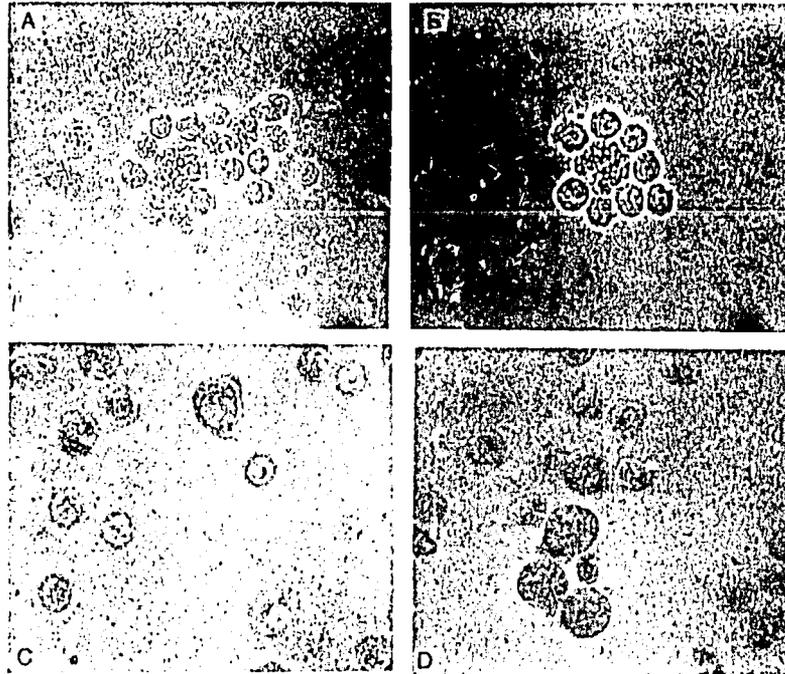


Figure 1. A) Rosette formation with *E. histolytica* HMI:IMSS trophozoites and HT-29 cells; B) in the presence of Sp2/0-Ag14 (C) and FIP1D5 (D) anti-*E. histolytica* monoclonal IgA.

Table 1. Inhibition of *E. histolytica* adherence to MDCK or HT-29 cell lines.

Cell line	Antibody	Adherence (%)	Inhibition adherence (%)
MDCK	-	80	-
	FIP1D5	13	85
	Hiperimmune Serum	0	100
HT-29	-	90	-
	FIP1D5	33	64
	serum	36	60

Affinity purity anti-monoclonal FIP1D5 was used at a protein concentration of 100 µg/ml. Ascitic fluid from Balb/c mice inoculated with Sp2/0-Ag14 cells, previously precipitated with saturated (NH₄)₂ SO₄ solution (50% v/v) was used at 100 µg/ml protein concentration. Anti-amoeba hyperimmune serum was diluted 1:10. 100 µl volumes were used in each case.

Table 2. Inhibition of *E. histolytica* adherence to fixed or mice gerbils cononic mucosa.

Animal	Inhibition of Adherence (%)			
	Ascitic ^a fluid	F1P1 ^b D4	F1P1 ^b D5	Hyperimmune serum ^c
BALB/c	-	40	49	74
Gerbils	-	41	67	48

a) Ascitic fluid treated as was indicated in Table 1, adjusted to a protein concentration of 90 µg/ml.

b) Affinity purity anti-*E. histolytica* monoclonal IgA (F1P1D5) (90 µl/ml).

c) Anti-amoeba hyperimmune serum was used as indicated in Table 1.

DISCUSSION

In the present study, experimental conditions were kept, similar to those described in other *in vitro* target cell systems.^{3,4} The HT-29 cell line was used as an *in vitro* adherence model since this line offers advantages over other epithelial cell lines. HT-29 cells are physiologically closer to normal target cells for amoebae.⁵ Our results indicate that this may be a useful model for adhesion studies of *E. histolytica* to human cells. There is a correlation between the *in vitro* model and the adherence model of trophozoites to glutaraldehyde fixed colonic mucosa, either from BALB/c mice or gerbils. Though these two species are different in susceptibility to amoebic infection *in vivo*,^{6,7} in the *in situ* model of adherence differences were not evident.

Our results suggest that although the biological significance of the secretory immune response in amoebiasis remains obscure, a reasonable possibility is that local immunity plays a critical role in maintaining the balance between intestinal and luminal amoebiasis. This may contribute to the differences in world prevalence and morbidity of the disease.

ACKNOWLEDGMENT

This work was supported by the Agency for International Development US AID through the grant No.936-5542-02-5239, 130.

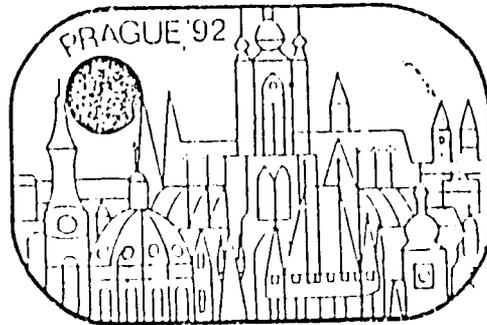
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ABSTRACT BOOK



AUGUST 16-20, 1992
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MOLECULAR ENGINEERING OF CHOLERA TOXIN

Burnette, W.N.,¹ and Kaslow, H.R.,² ¹Amgen Inc., Thousand Oaks, California USA; ²University of Southern California, Los Angeles, California USA.

Cholera toxin produces the major pathologic effects of *Vibrio cholerae* infection. It is an ADP-ribosylating toxin composed of a catalytic A subunit and a homopentameric B oligomer. Since A subunit enzyme activity is apparently essential for toxicity, its elimination should help to insure the safety of toxoid-based vaccines for cholera immunoprophylaxis. In order to produce an A subunit that lacks enzyme-related toxicity, yet is structurally competent to achieve holotoxin formation, we have utilized site-directed mutagenesis of the A subunit gene to functionally map this protein. A series of site-specific polypeptide analogs produced in recombinant *E. coli* have enabled us to map domains of the A subunit contributing to its catalytic activity and to its differential recognition of cellular substrates. Particularly noteworthy for the development of a "genetic toxoid" vaccine were the substitutions of Lys for Arg¹, Asn for His⁴, and Gln for Glu¹²; these single residue exchanges resulted in analogs lacking detectable ADP-ribosyltransferase activity. Modulation of cholera toxin function through rational modification of structure should contribute to our understanding of its competence as an immunogen and may provide for a safe yet more efficacious cholera vaccine component capable of eliciting a protective mucosal immune response.

HUMORAL IMMUNE RESPONSE AGAINST *S.typhimurium* ANTIGEN FRACTIONS AND PROTECTION

Calderón G.M. Pérez, C., Jiménez, C. and Meléndro, E.L.
Experimental Medicine Department, Faculty of Medicine UNAM.
México City, Mexico.

Balb/c mice were immunized with three oral doses of live *S.typhimurium* given in consecutive days (30C) or every week (30S). Control groups of non-immunized mice were included. In order to correlate the antibody response at intestinal and systemic level with protection, an oral challenge with a virulent strain of *S.typhimurium* was given. The group of mice 30S was better protected. We did not find correlation between antibody levels (IgG, IgM, IgA) against a proteic extract of *S.typhimurium* and protection. Using Western blot we found that the interval between doses affects the antigenic fractions recognized by the antibodies. Also there were differences between the fractions recognized by intestinal and serum antibodies, except for two bands of 88 kDa and 32 kDa that were recognized at a high frequency by both sources of antibody, and correlated better with the protection of animals.

Work supported by the grant No.936-5542-02-5239.130 from the Agency for International Development AID, USA

E.HISTOLYTICA ADHERENCE: INHIBITION BY IgA MONOCLONAL ANTIBODIES.

Ramon Fernando, Leyva Oscar, Rico Guadalupe, Morán Patricia, Melendro E.L., and Jiménez Cecilia, Experimental Medicine Department, Faculty of Medicine UNAM, Mexico City, México.

With the aim of investigate the role of anti-E.histolytica IgA in the interference with the adhesion process *in vitro* and *in situ*, anti-E.histolytica monoclonal IgA was used to determine the percent of inhibition of MDCK cells to E.histolytica HNI:1MSS virulent trophozoites. On the other hand, adherence of trophozoites to Balb/c mice and Gerbils meriones intestinal mucosa, were investigated in the presence or absence of anti-E.histolytica monoclonal IgA. Preliminary results suggests that F₁P₁D₅ monoclonal IgA, which is a dimeric IgA that recognized surface related epitopes, can interfere with adhesion mechanisms *in vitro* and *in situ*. Taking into account that amoebiasis is a disease primary adquired by the oral route, the study of a possible protective role of IgA in this disease may be improved with the utilization of monoclonal antibodies with the proper isotype.

Work supported by the grant No.936-5542-02-5239.130 from the Agency for International Development AID, USA

VECTOR-ENCODED IL-5 AND IL-6 ENHANCE SPECIFIC MUCOSAL IMMUNOGLOBULIN A REACTIVITY *IN VIVO*

Ramsay, A.J. John Curtin School of Medical Research, Australian National University, Canberra, Australia

IL-5 and IL-6 enhance the production of several Ig classes *in vitro*, however a major function may be to promote IgA synthesis, thereby playing a crucial role in regulating mucosal immunity. In order to study their influence *in vivo*, we have immunised mice with recombinant vaccinia viruses constructed to coexpress murine IL-5 or IL-6 and haemagglutinin (HA) of influenza virus. Numbers of anti-HA IgA antibody-secreting cells found in lungs of mice given VV-HA-IL5 or VV-HA-IL6 intranasally were markedly higher than in those given control virus, as detected by ELISPOT and *in situ* immunofluorescence. Preliminary data suggest that the vector-expressed cytokines may influence both T and B cell activity in this model. The potential of these constructs in enhancing protective immunity and in clarifying the role of cytokines in mucosal immunoregulation is being investigated.

IgA PRODUCTION AND TRANSPORT IN THE MURINE LIVER AFTER MUCOSAL IMMUNIZATION

Wu, H.-Y., White, P.L., Beagley, K., Jackson, G.D.F., Mostocky, J., and Russell, M.W. University of Alabama at Birmingham, Birmingham, Alabama, U.S.A., and University of New South Wales, Sydney, Australia.

Several recent studies indicate that the liver is a site where antibody-secreting cells congregate after immunization by peroral or parenteral routes. The profile of specific IgM, IgG1, IgG2a, and IgA antibody-secreting cells in the liver of mice resembles that of the spleen rather than the intestinal lamina propria, regardless of the route of immunization. Peroral immunization increases the proportion of specific IgA antibody-secreting cells in all three organs, but liver mononuclear cells contain a higher proportion of total IgA-secreting cells than spleen cells. Although IgA predominates in bile, IgM and IgG antibodies are clearly detectable after immunization, and specific activities of biliary antibodies are generally higher than in serum, implying their intrahepatic production. Immunization results in increased numbers of both B and T cells recoverable from the liver. The high ratio of IgG1:IgG2a subclasses among liver Ig-secreting cells suggests a preponderance of Th2 over Th1 activity. In support of this, liver T cells secrete relatively more IL-5 than spleen T cells, both spontaneously and upon mitogen stimulation. Mouse hepatocytes transport plgA into bile by means of polymeric Ig receptor (secretory component), whose expression may be subject to regulation by cytokines, such as IL-4. Implantation of IL-4-secreting transfectoma cells in mice appears to enhance hepatobiliary transport of plgA, and possibly also biliary and salivary antibody responses to immunization.

SPECIFIC ANTI *E.histolytica* IgA MONOCLONAL ANTIBODIES

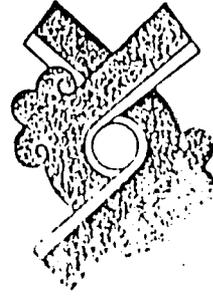
Ximénez Cecilia, Horán Patricia, Ramos Fernando, Leyva Oscar y Melendro E.L.

Experimental Medicine Department Faculty of Medicine, National University of Mexico, Mexico.

Anti-*E.histolytica* IgA monoclonal antibodies were produced to study their possible interference with some of the well-known pathogenic mechanisms of *E.histolytica* in vivo and in vitro. Balb/c mice were intragastrically and intraperitoneally immunized with membrane extract antigen or axenically cultured *E.histolytica* strain HMI:INSS trophozoites in the presence or absence of IgA antibody production adjuvants. Hybridization was performed using Sp2/OAg 14 myeloma cells and cells from Peyer's patches, or spleen (1:5 ratio). Anti-*E.histolytica* IgA parental clones were identified by the ELISA technique, and cloned by limiting dilution technique. Three clones were obtained with Peyer's patches cells which produce specific IgA and two IgG class antibodies. Indirect immunofluorescence studies indicated that clones F1P1D4 and F1P1D5 recognized surface associated epitopes. In the Western blot of *E.histolytica* antigen, they recognized a doublet of approximately 200 kDa and a 60 and 30 kDa molecules respectively.

Work supported by the grant No. 936-5542-02-5239.130 from the Agency for International Development AID, USA

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079-R EXPRESSION OF THE 65-KDA ANTIGEN OF *Mycobacterium leprae* FROM *E. coli* IN *Yersinia enterocolitica* CELL. Estrada-Gil, J. P., Lamb, J.L., Palacios-Lacort, G.P. & Colston, H.J. Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México, D.F. 04510. Leprosy and Mycobact. Res. Lab., NIMR, London.

The failure to grow *M. leprae* in vitro has hampered by restricted studies on the basic biology and immunology of this organism, which causes human leprosy. In recent years, the potential of recombinant DNA technology to provide proteins of *M. leprae* has begun to be exploited with the cloning of the *hly* gene into *E. coli* despite the relative difficulty of *E. coli* to express mycobacterial genes. The presence of the 65 kDa antigen of *M. leprae* was achieved from a λ -phage clone into the high-copy number plasmid pUC19. *E. coli* containing these recombinant plasmids have secreted the antigen. The ability of *E. coli* to recognize the hybrid translational start sites was confirmed by constructing additional clones in which the gene is flanked by transcription terminators from plasmid. The insert from the *hly* gene (H108, 562-636) in the 65 kDa antigen was gel-purified and resolved into the total site of pUC19 to produce plasmids pUC101, pUC102 and pUC103 with the insert in opposite orientations. The constitutive nature of expression of the gene in *E. coli* was demonstrated by the ability of the pUC103 sub-clones to synthesize the antigenic peptide terminators flanking the cloning site prevent transcription originating from promoter-active sequences on the plasmid.

Financiado de la OMS

080-R PURIFICATION OF LEISURE ANTIGENS FOR SEROLOGIC IDENTIFICATION OF MIBACULOSIS. Dinegs, O., Padilla, A., Hernández, M., Santos, M., Sotelo, J., Larralde, C., Górriz, I., and Scattolo, E. Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Instituto Nacional de Neurología y Neurocirugía, México, D. F., MÉXICO.

Purification of antigens for immunodiagnosis is mandatory to improve specificity. An electrophoretic procedure was used to separate the different proteins in *Leishmania crassiceps* vesicular fluid. 27 bands were identified: 17 having a molecular weight above 60 kD, and 10 under 60 kD. Among the low molecular weight proteins a group of them was diluted, with molecular weights between 8 and 14 kD, accounting for about 10% of the total protein content. This group of proteins does not seem to be glycosylated, since they did not bind any of several lectins. Preliminary results indicate that hyperimmune rabbit serum and several human sera from neurocysticercotic patients, have significant levels of antibodies against them. A more thorough evaluation of their potential use in immunodiagnosis is underway. Support by: IN 2074B9 DGAPA, UNAM, and DUBB LUNACYL.

081-R CHEMICALLY UNRELATED MYCOBACTERIAL PEPTIDES AS ANTIGENS AND COMPETITORS IN ANTIGEN RECOGNITION BY HUMAN T CELLS.

Paredes, M., Sandoval, L., and Lina, J. Instituto de Ciencias Biológicas, U.P.M., Cuzco y Plan de Ayala, México, D.F. 04510

Understanding resistance to mycobacterial infectious disease requires identification of antigens and epitopes (antigenic determinants) that stimulate cell-mediated immune responses. In this study, a PHA-restricted T cell epitope (peptide 1 through 20) of the 19-kD protein of *M. tuberculosis* was identified. Purified blood mononuclear cells from two MDR-TB patients with tuberculous response not only to the 19-kD immunoblot fraction of *M. tuberculosis* but also to the peptide 1-20. A *M. tuberculosis*-sensitive T cell clone isolated from one of the patients (clone 3) showed a strong proliferative response to the 19-kD protein and recognized the peptide 1-20. While failed to recognize a negative control peptide (peptide 45 through 65) of the 19-kD mycobacterial protein (peptide 45-65) or a negative control antigen (64-104). The antigen recognition to peptide 1-20 was shown to be PHA restricted. This PHA restriction was confirmed using a PHA-restricted mycobacterial T cell epitope as competitor. These results demonstrated that this mycobacterial competitor significantly reduced the antigen recognition of peptide 1-20. The results observed was dose dependent. This approach has permitted to identify a mycobacterial peptide competitor that can control significantly the activation of one antigenic epitope without affecting others, and represents the role of mycobacterial peptide competition as a helpful mechanism in the downregulation of mycobacterial antigen recognition.

082-R IMMUNE RESPONSE AGAINST *S. typhi* ANTIGEN FRACTIONS IN PATIENTS WITH TYPHOID FEVER.

Pérez, C., Calderón, G.M., Ramírez, C., and Meléndez, F.J. Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, Apdo. Postal 70-641, México D.F. 04510.

The proliferative responses of peripheral blood mononuclear cells and plasma antibodies of ten patients with typhoid fever, and normal individuals without history of typhoid fever or typhoid fever immunization, were analyzed using antigen fractions from two protein extracts of *S. typhi*. Fractions from each extract were separated by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose filters by electroblotting and processed to obtain Ag-bearing nitrocellulose particles for use in lymphocyte cultures. Although the individual proliferative responses were heterogeneous, we identified five immunogenic regions of 75-85 kDa, 57-58 kDa, 47-64 kDa, 18-22 kDa and 16-21 kDa. IgG and IgM anti-*S. typhi* were recognized at high frequencies bands of 90 kDa, 70 kDa and 30 kDa. The IgA antibodies recognized preferentially bands of 82-5) kDa and 48-65 kDa.

083-R TUBERCULOSIS VACCINE: EXPERIMENTAL EVALUATION OF PURIFIED ANTIGENS IN MURINE TUBERCULOSIS. Valdez, C., Espinoza, M., Hernández, J., and E. Scattolo. Instituto de Neurología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México, D. F., 04510, MÉXICO.

Vaccinations of mice with an A-typing extract from *Leishmania* sp. (L. crassiceps) against challenge with *L. crassiceps* was tested as successfully as did antigen extracts from *L. crassiceps*. Electroblotting techniques were employed to purified *L. crassiceps* antigen from the vesicular fluid. Twelve different clusters of antigens were individually evaluated in their ability of immunoprotection. For this purpose, ten mice (10 M) per group were used for each antigen. The immunoprotection was tested 10 weeks after challenge. Mice were challenged with the extract of each immunoprotective antigen. 10 days after infection and the extract that was determined individually. The results showed that *L. crassiceps* antigen could be classified in three types identified by their molecular weight: fast proteolytic antigens, vaccine protecting a type of antigens correlated to the parasite growth. Supported by: DGAPA, UNAM, (CONCYT).

084-R

CORRELATION BETWEEN ANTITETANUS ANTIBODY LEVELS IN GUINEA PIG SERA MEASURED BY TOXIN NEUTRALIZATION TEST IN MICE AND ELISA. Pasetti, M.; Brero, M.L.; Geluchli, S.; di Paola, C.; Eriksson, P.; Mathet, V.; MANZINI, R. IDHU, Univ. de Bs. As. Junín 956 (1113), Buenos Aires Inst. Nacional de Microbiología Avda. Vélez Sarsfield 561 (1281), Bs. As.

Different bioassays are used to estimate activity of tetanus vs. toxin. Challenge tests and the measure of antibody levels present in sera of vaccinated animals are the most used. At present, in Argentina, the activity of tetanus vaccines is estimated by Toxin Neutralization Test (TNT). In mice, measuring tetanus antibody levels in immunized guinea pigs sera. This test has several disadvantages: it is expensive, labor intensive and require a large number of animals. Activity estimation is influenced by strain, diet and maintenance conditions of the animals. Moreover, the use of animals has also caused concern in developing countries. It is desirable to develop in vitro tests. As a partial step in the development of an alternative method to evaluate activity of tetanus vaccines, we report an indirect ELISA to measure antitetanus antibody levels in guinea pigs sera. 50 serum samples from guinea pigs immunized with 0.1, 0.5, 1.0 and 2.0 A

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055-F

RECOGNITION OF ADULT WORM AND MUSCLE LARVAE ANTIGENS DURING THE COURSE OF HUMAN TRICHINELLOSIS. Salinas-Tobón, M.B., Chaparrón, M.B., Ricardos, J. and Ortega-Pierron, M.C. Depto. Inmunología, ENEC and Depto. Genética y Biología Molecular, CINVESTAV-IPN, MEXICO, D.F.

Recently, human outbreaks of trichinellosis have increased in Mexico. Although in some cases the infection by *T. spiralis* has been confirmed there is still a need for the development of more accurate and complete diagnostic assays which include detection of antibodies (Ab) to all stages of the parasite. Thus these assays may have prognostic as well as diagnostic value. In this context, we carried out a longitudinal epidemiological study in humans infected with *T. spiralis* to identify adult (AD) and muscle larvae (ML) antigens that were recognized during the infection. A total of 88 sera from 22 persons obtained from an outbreak of trichinellosis were analyzed by ELISA, Western Blot (WB) and coprecipitation of surface labeled antigens at 3, 5, 7, 15, 31 and 57 weeks post-infection. Ab to AD antigens were detected by week 3 while response to ML components was observed by week 5 in 100% of patients. The Ab response increased by weeks 5 and 7 and lower Ab titres were observed by week 15 to AD antigens and by week 57 to ML molecules. WB analysis revealed recognition of several AD and ML components by sera of these patients. The most frequently detected AD antigens had a M.W. of 105, 76, 67, 53, 44 and 34 kd while components of 98, 67, 63, 55, 47, 43 and 41 kd from ML were mainly detected by sera samples. Coprecipitation and SDS-PAGE analysis of radiolabeled ML proteins showed recognition of all surface labeled components of this parasite. M.W. of such proteins were similar to some of the ML antigens detected by WB analysis. All these results suggest that differential recognition of stage specific parasite antigens may be useful to develop more reliable methods with prognostic and diagnostic value for human trichinellosis.

056-F

USE OF POLYCLONAL SERA TO DETERMINE RESPIRATORY SYNCTIAL VIRUS ANTIGEN. Viraño, R., Bustos, J. and Córrea, C. Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, Apartado Postal 70641, 04510 México, D.F.

Respiratory syncytial virus (RSV) antigen is usually identified by immunochromatographic techniques: immunofluorescence or enzyme immune assay (EIA). Although these techniques are rapid and easy to perform they have some drawbacks. The processing of the samples and the interpretation of the results with fluorescein labeled immunoglobulin require skilled personnel and monoclonal antibody. An specific EIA assay requires either antibody from two different species (sandwich) or monoclonal antibody. In this study we describe the use of polyclonal sera in EIA to determine RSV antigen from either viral suspensions, infected cells or proteins extracted from infected cells. Viral suspension with titer of 10^6 plaque forming units/ml (PFU/ml) infected cells Vero or Hep-2 (24 h post-infection); multiplicity of infection 0.8 or protein extracted from 4×10^6 infected cells (5.5 mg of protein) were added per well. Sera with titer of 4×10^6 plaque reducing units/ml, and peroxidase labeled anti-IgG were added. RSV infectivity expressed in PFU was correlated linearly to the specific optical density obtained by EIA.

057-F

CELLULAR RESPONSES OF THE PLETHRA PATCHES AND SPLEEN IMMUNE RESPONSE TO *Entamoeba histolytica* AFTER ORAL AND MUCOSAL STIMULATION. Muñoz-Hernández, L., Enriquez-Rincón, P. and Campos-Rodríguez, P. Departamento de Biología Celular, CINVESTAV-IPN, México, D.F. 01000.

The local induction of humoral immune response to *E. histolytica* has not been studied. This work has examined the kinetics of both mucosal and systemic immune responses of antibody producing cells (APC) to *E. histolytica* in Peyer's patches (PP) and spleen in Balb/c mice after intragastric (IG), rectal (R), and intraperitoneal administration (IP) of *E. histolytica*-glutaraldehyde fixed zymosanites (ZMF). Mice were given half a million of ZMF by the mentioned routes and sacrificed on days 3, 7, 9, 11 and 13 after stimulation. We also studied IgM, IgG and IgA APC responses to *E. histolytica*, in mice receiving the antigen ZMF four times once a week, and sacrificed 7 days after the last administration. The number of APC to *E. histolytica* was assessed by the technique of ELISPOT in nitrocellulose membranes. The APC response to *E. histolytica* was detected in both PP and spleen with the three routes, indicating that either mucosal or systemic stimulation by ZMF generates both systemic and mucosal responses. In relation to the kinetics, maximal APC response to *E. histolytica* occurred at day 7 in PP when either the IP or IG route was used, whereas in spleen the maximal APC response occurred on day 6 or on day 7, when IP or IG route was used respectively. Spleen responses were higher than those detected by when the route was IP, but they did not show difference when the route was IG. With rectal inoculation, the maximal APC response to *E. histolytica* was detected on day 9 in PP, although this data could not be established in spleen due to variations in response observed by this route.

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058-F

HUMERAL IMMUNE RESPONSE TO PROTEIN ANTIGENS OF *S. typhimurium* AND PROTECTION.

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Balb/c mice were immunized with three oral doses of live *S. typhimurium* given in consecutive days (3DC) or every week (3W). Control groups of non-immunized mice were included. In order to correlate the antibody response of intestinal and systemic level with protection, an oral challenge with a virulent strain of *S. typhimurium* was given. The group of mice 3W was better protected. We did not find correlation between antibody levels (IgG, IgM, IgA) against a protein extract of *S. typhimurium* and protection. Using Western blot we found that the interval between doses affects the antigenic fractions recognized by the antibodies. Also there were differences between the fractions recognized by intestinal and serum antibodies, except for two bands of 88 kDa and 31 kDa - that were recognized at a high frequency by both sources of antibody, and correlated better with the protection of animals.

059-F

ESTABLISHMENT OF PERSISTENT INFECTED MAIPOPHAGE CELL LINE BY RESPIRATORY SYNCTIAL VIRUS. Bustos, J. and Córrea, C. Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, Apartado Postal 70641, CP 04510, México.

Respiratory syncytial virus is the most important cause of acute viral lower respiratory diseases in infants and preschool children. In adults, particularly in older subjects, it might originate severe pneumonia and adult respiratory distress syndrome. Furthermore, in immune suppressed individuals it originates persistent infection. Circumstantial evidences suggest that persistent infections might be associated with chronic disease as asthma. The knowledge about the mechanism by which persistent infection is established and maintained, and the cell type selected for the virus to persist is limited. However, those questions can be studied in experimental models in vitro with persistent infected cell cultures. Herein we report the establishment of a long term infected murine macrophage cell line (F3R01) with a human RSV strain (long). The highest efficiency of persistent infected cells was obtained with a multiplicity of infection of 0.1. Viral persistence was confirmed by determining extracellular virus and cell viral antigens in various subculture cells. Subcultures viral titres expressed in plaque forming units (PFU/ml) fluctuated between 10^4 and 10^7 viral particles per cell.

060-F

EFFECT OF THE MULTIPLICITY OF INFECTION ON RUBELLA PERSISTENCE. Sagmeister, B.

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Rubella virus (RV) can originate persistence infection in individuals infected congenitally and perinatally. In both cases a chronic overt disease might develop. Although the mechanism the virus uses to persist is unknown, circumstantial evidences suggest the involvement of antiviral antibody. Our purpose was to study how anti-rubella antibody might participate in the establishment and maintenance of persistent infection. We decided to develop an experimental model in vitro. A murine macrophage cell line (F3R01) was infected at different multiplicities (moi): 0.5 and 10 with and without 10^6 U.I. Viral persistence was confirmed by determining extracellular virus, viral antigen and infecting centers on the infected surviving cells after subculturing 20 times. Viral titres of 10^4 to 10^7 were obtained when a moi of 0.5 was used, with moi of 10, values between 10^4 to 10^6 were obtained. In all the persistent cultures that were studied viral antigens were found. The efficiency of establishment of persistence infection was higher when the infection was done in the presence of 10^6 U.I.

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Original Article

Local and Systemic Antibody Response in Balb/c Mice Immunized with *Entamoeba histolytica* Trophozoites¹BERTHA B. VILLAREAL,¹ EMMA E. MELFENDRO,^{1,2} FERNANDO RAMOS,^{1,2} and CECILIA XIMENEZ,^{1,2}¹ Faculty of Chemical Sciences, University of Simolot, Culiacán Simolot, México.² Experimental Medicine Department, Faculty of Medicine, National University of Mexico, Mexico, City.

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Abstract

Several immunization schedules with *E. histolytica* trophozoites were tested on Balb/c mice in order to induce antibody responses, both in intestinal secretions and in serum. Mice were immunized either orally, systemically, or using one of two combined schedules: the oral route followed by the systemic route (footpad), or vice versa. Each of the immunization schedules used in this project induced an anti-*E. histolytica* antibody response and there appears to be a correlation between the immunization route employed and the immunoglobulin isotype induced in the gut. Secretory IgA production is favored by the oral administration of trophozoites, whereas mucosal IgG appears to be enhanced by the systemic immunization route. Both schedules are effective in the induction of secretory IgA in the gut,

yet higher and earlier levels of IgA appear in orally immunized mice. When systemic immunization is employed, the increase in antibody levels in the intestinal fluid is slower, and IgG is the predominant class. The combined oral/systemic routes of immunization appear to be comparably effective for the induction of local and systemic IgA and IgM antibody production. However, mice immunized first systemically and then locally produce more IgG in both compartments. Combined schedules modify the isotype pattern of antibody responses in serum and in intestinal secretions when compared with single (i.e., oral or systemic) schedules, but they do not appear to favor a secretory IgA immune response. (*Arch Med Res* 1992; 23: page 69)

KEY WORDS: *E. histolytica*, intestinal and serum antibody responses; IgG; IgA; IgM

Introduction

Amebiasis is an infection caused by the ingestion of *Entamoeba histolytica* cysts. The establishment of amebic disease depends ultimately on the outcome of the host-parasite relationship in the intestine. There is still controversy over the biological relevance of local and circulating antiamebic humoral antibodies. Much *in vitro* work has been done on the effects of humoral antibodies on *E. histolytica* (1, 2), as well as on the elaborate mechanisms played by the parasite to evade them (3, 4). It has been difficult, however, to define the

role of these antibodies *in vivo* as frequent reinfections occur in the presence of high antibody titers (5) and such high titers sometimes coincide with the acute stage of the disease (6). Only isolated reports have implicated serum antibodies in the protection against this disease (7).

Secretory immunoglobulins constitute one of the first lines of defense against microorganisms in mammals (8). IgA, the predominant immunoglobulin in human secretions, appears to block adhesion, therefore interfering with invasion of deeper tissues by microorganisms (8). Although little is presently known about the biological significance of the secretory immune response in amebiasis, it is fair to speculate that, as in other systems, IgA may play an active role in local immunity against *E. histolytica* (9).

In order to study the effect of local and systemic routes of immunization (or combination of both) on the appearance and the isotype of the antibodies induced, we tested the serum and the intestinal fluid of immune Balb/c mice for the presence of

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anti-*E. histolytica* antibodies of the IgG, IgM and IgA classes after such immunization schedules. Gladhim and Kongshavn have reported that Balb/c mice are genetically susceptible to experimental *E. histolytica* infections, even though mice in general have not been found to particularly susceptible to this parasite (10).

Materials and Methods

Microorganisms. *E. histolytica* HM1:IMSS were axenically cultivated in TYE-S-33 medium (11). Cultures were incubated at 36.8°C and trophozoites were harvested during the logarithmic growth phase. They were washed three times with cold 19 mM potassium phosphate buffer (PPB) pH 7.2 containing 0.27 M sodium chloride (NaCl) and subsequently centrifuged at 200 g for 10 min at 4°C and resuspended in PPB or 0.5 M sodium bicarbonate solution (SBS).

Immunization Procedures. Groups of ten Balb/c mice each were immunized using one of four different schedules: Group I: three doses of orally administered 4.5×10^6 trophozoites suspended in 0.2 ml of SBS, on days 1, 2 and 9. Group II: two doses of 1.5×10^6 trophozoites suspended in 0.05 ml of PPB injected subcutaneously (SC) into the footpad on days 1 and 8. Group III: three oral doses (4.5×10^6 trophozoites) on days 1, 2 and 9, followed by two SC doses (1.5×10^6 trophozoites) on days 16 and 23. Group IV: two SC doses (1.5×10^6 trophozoites) on days 1 and 8, followed by three oral doses (4.5×10^6 trophozoites) on days 15, 16 and 23. Control mice for each experimental group were inoculated on the same days and with the same volume, but with PPB or SBS as required.

Collection of Serum and Intestinal Fluid. Mice were bled from the retroorbital plexus, the serum was separated by centrifugation and was stored at -20°C until processed for antibody measurement. Mice in groups I and II were sacrificed 14 or 30 days after the first immunization and mice from groups III and IV were sacrificed 7 days after the last immunization.

Fluid was collected from the small intestine using a modified version of the method of Elson et al. (12). The entire intestine was removed and, after closing one end, 2 ml of 0.02 M phosphate buffer solution (PBS) pH 7.4 was injected into the lumen. The intestine was gently massaged (ten times) and the resulting fluid was collected in a test tube. The fluid was then centrifuged at 1000 g, for 30 min at 4°C. Supernatant fluids were collected and soy bean trypsin inhibitor (Sigma Chemical, St. Louis, MO, USA) (200 µl of 1.0 mg/ml), ethylene diamine tetraacetic acid (EDTA) (Sigma Chemical, St. Louis, MO, USA) (20 µl, 50 mM) and phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical, St. Louis, MO, USA) (20 µl, 100 mM) were added to inhibit protease activity. The mixture was subsequently centrifuged at 1000 g (20 min, 4°C), the supernatant fluid was separated and PMSF was added and kept at room temperature for 5 min. Finally, bovine serum albumin (BSA) (Sigma Chemical, St. Louis, MO, USA) (50 µl of 200 mg/ml) was added and the mixture stored at -20°C until used.

Measurement of Anti-*E. histolytica* Antibody

Anti-*E. histolytica* antibodies were measured in both serum and in intestinal fluid by the ELISA technique (13), using

Remove-A-Well Immunolon H strips (Dynatech Laboratories Inc., Alexandria, VA, USA). Wells were coated with 10^6 *E. histolytica* HM1:IMSS trophozoites suspended in carbonate buffer (50 µl, 0.01 M pH 9.6). Strips were dried under vacuum conditions at room temperature. When ready for use, the wells were rehydrated with 0.02 M PBS, pH 7.2 added with 0.5% Tween 20 (Sigma Chemical, St. Louis, MO, USA) and 0.5% BSA (PBS-TW-BSA) and washed twice with PBS-TW. To block the sites not covered with trophozoites, wells were filled with a 3% solution of BSA in PBS and were incubated overnight at 4°C and washed again twice with PBS-TW. Wells were charged with 50 µl of the test sample and were incubated for 60 min at room temperature. They were washed afterwards three times with PBS-TW-BSA and twice with PBS-TW. Fifty microliters of anti-mouse IgG, IgM and IgA heavy-chain specific antibodies coupled to peroxidase (Cappel, Malvern, PA, USA) were added at the adequate dilution to each sample and the mixture was incubated at room temperature for 60 min. The plates were washed as before and 50 µl of the substrate (10 ml of 0.1 M citrate buffer, pH 4.5, containing 10 mg O-phenylenediamine) (Sigma Chemical, St. Louis, MO, USA) and 4 µl of a 30% solution of H_2O_2 were added.

Preliminary studies were undertaken on fluid and serum samples from several successfully immunized animals in order to determine optimal dilution for routine assays. Serial specimen dilutions were tested by ELISA, and semilog plots of the reciprocal of dilution against absorbance for each specimen were drawn. An extended linear relation between the optical density (O.D.) values and the logarithm of the sample dilution was calculated. Appropriate dilutions were chosen taking into account those lying on the linear portion of the curves. Serum samples were diluted 1/100 for IgM and IgA and 1/1000 for IgG determinations. The IgG, IgM and IgA class specific antibodies were titrated so as to give comparable O.D. values when reacting with a known amount of the corresponding purified immunoglobulin (14).

Results are expressed as mean O.D. values of experimental groups after subtracting the mean O.D. control values.

Statistical Analysis

Statistical analysis was performed by Ms. Erika Pallares of the Unidad de Cómputo, Facultad de Medicina, UNAM. Significant differences between groups was established by computer-assisted analysis of variance. The significance of differences between means in each isotype studied was determined by Student t test when variances were homogeneous, and by Mann-Whitney U test when variances were heterogeneous (15).

Results

Anti-*E. histolytica* Antibody Response

All the immunization schedules employed in these experiments induced anti-*E. histolytica* antibody responses. Control (i.e., non-immunized) mice did not reveal anti-*E. histolytica* antibodies in serum or intestinal fluid.

Systemic Antibody Response

On day 14, levels of circulating IgA antibodies disclosed no significant difference between the two immunization routes

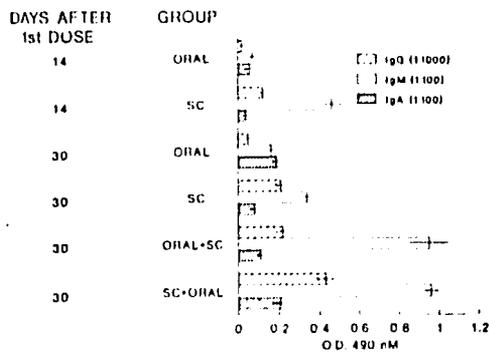


Figure 1. Sera from immunized Balb/c mice were tested for anti-*E. histolytica* antibodies of IgG, IgM and IgA classes using the ELISA technique as described in Materials and Methods. Values are the mean of O.D. at 490 nm + the standard error of the mean (SEM) of three separate experiments.

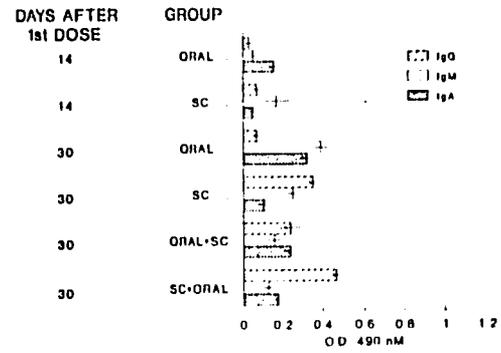


Figure 2. Intestinal fluid from immunized Balb/c mice were tested for anti-*E. histolytica* antibodies of IgG, IgM and IgA classes using the ELISA technique as described in Materials and Methods. Values are the mean of O.D. at 490 nm + the SEM of three separate experiments.

(oral or SC). However, at that point of time, the SC route was more effective than the oral route in inducing anti-*E. histolytica* antibodies of the IgG and IgM classes ($p < 0.001$) (Figure 1). The anti-*E. histolytica* antibodies induced by oral immunization appeared later in serum and, by day 30, the IgA antibody levels in this group were significantly higher ($p < 0.0001$) than in the SC immunized mice.

Comparing the two combined schedules of immunization, it was observed that both were effective in the induction of serum IgG, IgM and IgA. IgM levels appeared to be favored by such combined schedules, as compared to the single schedules (i.e., oral or SC) and the SC/oral immunization schedules specifically appeared to induce higher levels of IgG ($p < 0.005$) and IgA antibodies ($p < 0.05$). In terms of amount of serum anti-*E. histolytica* antibodies, the IgG class was the most abundant. Since serum dilutions used in the ELISA test were tenfold larger (10^5) than those used for IgM and IgA (10^3) detection (Figure 1).

Local Antibody Response

At the intestinal level, significant differences were observed between the orally and SC immunized mice at day 14 after the first immunization. IgA was the leading immunoglobulin in the orally treated mice when compared with the systemically (SC) immunized animals, where IgM was in turn the predominant class ($p < 0.005$) (Figure 2).

On the other hand, on day 30 after the first immunization, a change in the predominance of immunoglobulin class was observed: IgM and IgA antibody responses were significantly higher in the oral group than in the SC group ($p < 0.001$) (Figure 2), whereas the SC route of immunization appeared to be more effective than the oral route in the induction of anti-amebic IgG ($p < 0.001$) (Figure 2).

Combined immunization schedules were equally effective in the induction of IgM and IgA isotypes, although the SC/oral schedule induced higher levels of IgG in the intestinal fluid than the oral/SC schedule ($p < 0.01$).

Discussion

Oral, subcutaneous or combined schedules of immunization of Balb/c mice with *E. histolytica* HM1:IMSS trophozoites induce antibody production in both serum and in intestinal secretions.

There is a correlation between the route of immunization employed and the isotypes of the antibodies in the gut. IgA production at local level is favored by the oral administration of trophozoites, while local IgG appears to be favored by systemic immunization (Figures 1 and 2). Even though both schedules are effective for the induction of IgA antibodies in the gut, higher levels of this class appear earlier in orally immunized mice than in SC immunized mice (Figure 2). Although the appearance of circulating antiamebic antibodies is considered to be a consequence of a tissue invasion (16), this is apparently not a requisite for the induction of a local antibody response (17). In the present study mice were immunized intragastrically with axenically cultivated trophozoites (which is not the infective form of this parasite). Thus it appears possible to induce circulating antiamebic antibodies using the oral route of immunization and in the absence of intestinal tissue invasion, as trophozoites suspended in SBS lose their viability in a matter of minutes (18).

Intestinal antibodies are locally produced following oral immunization schedules (19). It is possible that a gradual systemic immune stimulation occurs as a result of migration of antibody-forming cells to the draining lymph nodes, or due to the absorption of soluble antigen into the circulation (20, 21). In rats, a sonicate of *E. histolytica* inoculated intraperitoneally or even directly into the Peyer's patches leads to the appearance of *E. histolytica* antibody-forming cells, first in the spleen and only later in the intestinal mucosa (22).

The systemic route of immunization also appeared to be effective in the induction of antibodies both in serum and in the intestinal secretions. It can be claimed that antibodies in the intestinal fluid may derive from serum, as suggested by other

models (23). In such cases, however, serum and intestinal levels of the respective classes usually correlate, and this was not the case in our study (Figures 1 and 2).

The optimal method for inducing a specific mucosal secretory IgA response thus remains to be defined. A central issue is the relative efficacy of parenteral vs. mucosal routes of immunization. Local immunization favors the stimulation of IgA B cell precursors in submucosal lymphoid follicles, where such cells are abundant anyhow (24). In contrast, and even though systemic immunization (24) or the combined local and systemic immunization schedule (25) appear to evoke an efficient mucosal IgA response, some reports claim that the systemic route is ineffective (26), or may even be suppressive of an IgA mucosal response (27).

Our results using the two combined routes of immunization demonstrate that both schedules (oral/SC and SC/oral) are effective for the induction of systemic and secretory IgA (and IgM) antibody production. Interestingly, mice immunized first systemically and then locally (i.e., orally), produce more IgG in both compartments. The systemic route was not found to be suppressive in our model, yet the oral route proved to be the better one to induce IgA at a local level.

Combined routes of immunization may modify the pattern of immunoglobulin responses in serum and in intestinal secretions when compared with the single routes at corresponding times (Figures 1 and 2). However, combined schedules do not appear to favor a secretory IgA immune response in the gut when compared with the oral route schedule alone. These observations may be important in designing the best immunization schedule to improve secretory IgA antibody responses in mice. Finally, caution has to be exercised before extrapolating these results to other animal species, as they may have a different immunological behavior towards this protozoan.

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