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"INMUNIZACIÓN ORAL CON ANTÍGENOS DE *Entamoeba histolytica* CLONADOS A TRAVÉS DE ANTICUERPOS IgA SECRETORES"

T E S I S

QUE PARA OPTAR POR EL GRADO DE

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DISCONTINUA

Dedicatoria

A mi madre, la mujer que ha sentado las bases sobre las que se sustentan todo lo que he logrado.....

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RESUMEN

La infección intestinal con *Entamoeba histolytica* genera una respuesta local con producción de anticuerpos IgA secretorios (IgAs) específicos que pueden bloquear la adherencia de los trofozoítos a líneas celulares derivadas de mamíferos. En este trabajo se identificaron varios antígenos de *E. histolytica* reconocidos por anticuerpos IgAs, a través de un inmuno-tamizaje de una biblioteca de cDNA con salivas de pacientes con amibiasis intestinal y absceso hepático así como saliva de un portador asintomático de quistes. Los antígenos identificados incluyen dos proteínas bien conocidas y candidatos a vacuna: la proteína rica en serinas (SREHP) y la proteína de 29 kDa rica en cisteínas (CREHP), así como cinco proteínas intracelulares conocidas en la amiba y tres no reportadas con anterioridad: la ciclofilina (EhCyp), la proteína ribosomal L-23a (EhRP-L23a) y un antígeno rico en ácido glutámico que no tiene homólogos en la base de datos de proteínas (EHSalJC1). La EhCyp se caracterizó y se determinó el efecto de su ligando, la ciclosporina A, sobre la proliferación de los trofozoítos tanto *in vitro* como *in vivo*. Los análisis de Northern blot mostraron un único transcrito para la EhCyp y EhRP-L23a, y al menos 4 transcritos para la EHSalJC1. Un estudio inicial de protección contra la infección intracecal de ratones C3H/HeJ, se realizó por inmunización oral de los ratones con péptidos recombinantes de la CREHP y EHSalJC1 administrados con toxina del cólera. A diferencia de los ratones no inmunizados, los inmunizados y en especial aquellos con CREHP, mostraron lesiones pequeñas ó totalmente ausentes en cortes histológicos de los ciegos, así como claras diferencias en la apariencia externa y en su contenido. Se observó una buena correlación entre el desarrollo de respuestas inmunes secretorias y sistémicas anti-*E. histolytica* con el nivel de protección observada. Estos resultados sugieren que los antígenos de trofozoítos que inducen respuestas secretorias en humanos, podrían ser utilizados en el desarrollo de una vacuna oral contra la amibiasis.

ABSTRACT

Intestinal infection by *Entamoeba histolytica* induces a local response with production of specific secretory IgA (sIgA) antibodies which can block adherence of trophozoites to mammalian cells. Several antigens of *E. histolytica* recognized by sIgA antibodies, were identified by immunoscreening of a cDNA library with saliva from patients with intestinal and hepatic amebiasis and saliva from an asymptomatic cyst carrier. The antigens identified include two well known proteins and vaccine candidates: the serine-rich (SREHP) and the 29 kDa cysteine-rich (CREHP) proteins, as well as five intracellular proteins of ameba and three previously unreported proteins of *E. histolytica*: cyclophilin (EhCyp), the ribosomal L-23a protein (EhRP-L23a) and a glutamic acid-rich antigen (EHSalJC1) which has not homologues in the protein databases. EhCyp was characterized and the effect of its ligand, cyclosporine A, on trophozoites proliferation *in vitro* and *in vivo* determined. Northern blot analysis revealed single transcripts for EhCyp and EhRP-L23a, and at least four transcripts for EHSalJC1. An initial protection study against intracecal infection on C3H/HeJ mice was carried out by oral immunization with recombinant peptides of CREHP and EHSalJC1 administered with cholera toxin. In contrast with the non-immunized mice, the immunized mice and specially those immunized with CREHP, showed small lesions or were totally absent in tissue sections of the ceca, as well as notorious differences in its external appearance and content. A good correlation between the development of secretory and systemic anti-*E. histolytica* responses with the level of protection was observed. These results suggest that the trophozoite antigens which induce secretory responses in humans, could be used in the development of an oral vaccine against amebiasis.

INTRODUCCIÓN

La enfermedad

La amibiasis, causada por el parásito protozoario *Entamoeba histolytica*, es considerada actualmente como la tercera causa de muerte por parásitos a nivel mundial detrás de la malaria y la esquistosomiasis. Se estima que el parásito infecta al 10% de la población mundial, de los cuales sólo el 10% desarrolla alguna modalidad de amibiasis invasora causando entre 40,000 a 100,000 muertes anuales, principalmente en países en vías de desarrollo con condiciones socio-económicas pobres, hábitos poco higiénicos y malnutrición. Inclusive algunos autores se refieren a la amibiasis como uno de los denominados "síndromes de la pobreza". La amibiasis invasora es uno de los principales problemas de salud pública en ciertas regiones de Asia, Africa y América Latina (Walsh, 1986). En México, la amibiasis ocupa el primer orden de mortalidad por parásitos. Entre un 20 y 25% de la población podría estar infectada, de los cuales más del 8% ha sufrido por lo menos un episodio de amibiasis invasora como se determinó por ensayos de hemaglutinación indirecta en la última encuesta seroepidemiológica nacional (Caballero y col., 1994). El estudio realizado sobre casi 70,000 sueros provenientes de toda la República Mexicana sugiere, con respecto a la anterior encuesta nacional (Gutiérrez y col., 1976), que la incidencia de amibiasis invasora se incrementó en las últimas dos décadas en más del 2%. Sin embargo, y debido al desarrollo de tratamientos más eficaces, el nivel de mortalidad debido al parásito ha disminuido significativamente. Paradójicamente y debido a la intensa explosión demográfica a nivel mundial, a pesar de que el número de casos fatales por persona ha disminuido considerablemente, los niveles de morbilidad y mortalidad se siguen manteniendo (Walsh, 1986).

En términos generales, el concepto de enfermedad causada por *E. histolytica* incluye: la colonización del intestino por amibas de esta especie, contacto íntimo o adherencia a la mucosa intestinal, ruptura de las barreras intestinales por enzimas o productos tóxicos liberados y lisis de las células epiteliales e inflamatorias del hospedero. En algunos casos,

que representa aproximadamente el 10 % de la población enferma, los trofozoítos penetran profundamente a la submucosa alcanzando las vías circulatorias mesentéricas e invadiendo órganos distantes, principalmente el hígado (Ravdin, 1986). El absceso hepático amibiano es la principal causa de muerte por esta parasitosis, sin embargo con poca frecuencia, las amibas en calidad de invasoras pueden afectar otros órganos como cerebro, pulmón, genitales y piel (Reed, 1992).

Los síntomas de la enfermedad gastrointestinal pueden ir desde ligero dolor abdominal con diarrea leve, hasta formas graves con dolor agudo, moco y sangre en las heces, fiebre, tenesmo y a veces ameboma o apendicitis. En el caso del absceso hepático se produce dolor intenso en el área abdominal con todas las manifestaciones clínicas, en mayor ó menor grado, de un proceso que afecta el hígado.

El parásito

Biología

Entamoeba histolytica es un parásito protozoario considerado como un prototipo de célula eucarionte muy primitiva. Taxonómicamente, se la clasifica de la siguiente manera (Zaman-Vigar, 1979):

Phylum	*Protozoa*
Subphylum	*Sarcomastigophora*
Superclase	*Sarcodina*
Clase	*Rhizopodea*
Familia	*Entamoebidae*
Género	*Entamoeba*
Especie	*histolytica*

El parásito oscila entre dos estadios de vida: el trofozoíto y el quiste. El trofozoíto, la forma móvil e invasora, mide 25 µm en promedio (Fig. 1); carece de mitocondrias, de aparato de

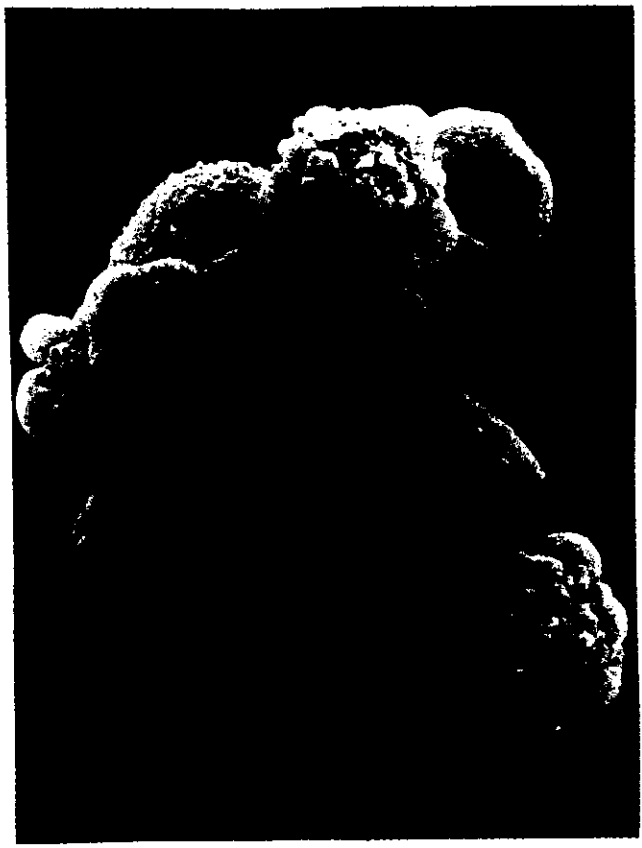


Fig. 1 Micrografía electrónica de barrido de un trofozoíto de *Entamoeba histolytica* fagocitando de manera simultánea varias células epiteliales en suspensión. El trofozoíto es la forma móvil e invasora de la amiba capaz de digerir casi cualquier célula que se cruce en su camino (Martínez-Palomo, 1987).

Golgi, de retículo endoplásmico y de lisosomas primarios como secundarios. A pesar de que el trofozoíto posee un citoesqueleto poco organizado es extremadamente móvil, desplazándose rápidamente a través de la emisión de unas proyecciones citoplasmáticas denominadas pseudópodos y filopodios. El parásito es capaz de ingerir una amplia variedad de partículas incluyendo bacterias y eritrocitos así como de destruir casi toda célula o tejido que se encuentra a su paso, de ahí su nombre de histolytica. El trofozoíto durante el movimiento presenta claramente dos zonas: una hialina asociada al sitio del desplazamiento denominada ectoplasma y otra con gran cantidad de corpúsculos, gránulos y vacuolas denominada endoplasma. Su membrana plasmática de 10 nm de ancho es similar a la de la mayoría de las células, con proteínas embebidas en una bicapa lipídica y rica en carbohidratos. La amiba al desplazarse presenta en la parte posterior una región denominada uroide, que corresponde al sitio hacia donde se mueven los receptores de superficie durante el fenómeno conocido como "capping". El capping le permite al trofozoíto desprenderse de moléculas que interaccionan con proteínas de su superficie como lo son los anticuerpos, constituyendo uno de los principales mecanismos de evasión inmune con que cuenta. El trofozoíto presenta además un sólo núcleo generalmente céntrico con cromatina periférica condensada en la cara interna de la membrana nuclear y un endosoma central similar a un nucleolo (Fig. 2) (Martínez-Palomo, 1987).

Por otra parte, el quiste que es la forma infectante y de resistencia de *E. histolytica*, mide entre 5 y 20 µm de diámetro (Fig 3). Dependiendo de su estado de madurez puede presentar entre 1 y 4 núcleos, idénticos en aspecto al de los trofozoítos. La principal característica estructural del quiste, es la presencia de una capa de recubrimiento de quitina que le confiere al parásito resistencia al medio ambiente y a la acción ácida de los jugos gástricos durante su paso por el estómago (Smith y col., 1979).

Organización genómica

En la amiba la división nuclear se presenta sin desintegración de la membrana, por lo que la condensación cromosomal no es evidente (Meza y col., 1990). Se desconoce el

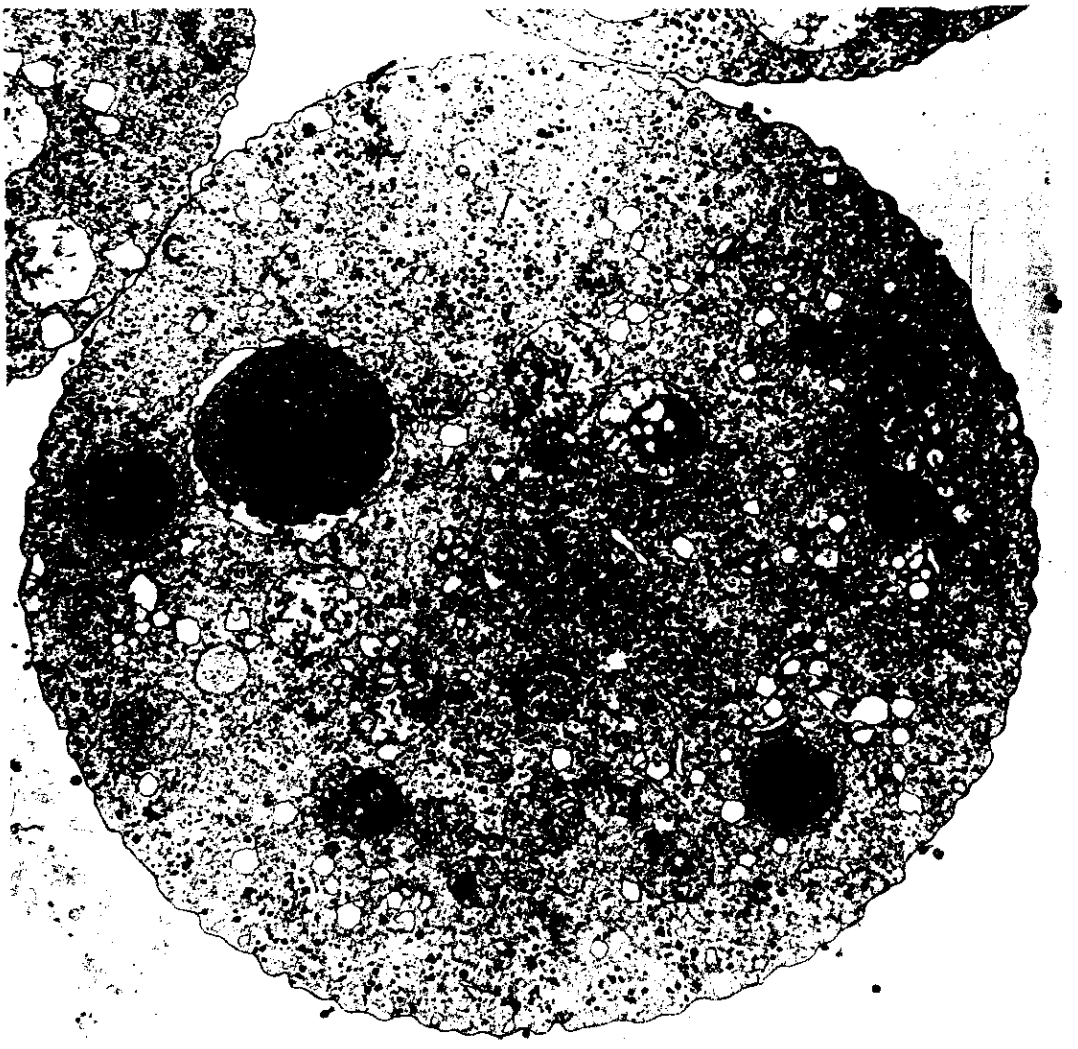


Fig. 2 Trofozoíto de *Entamoeba histolytica* visto bajo el microscopio electrónico. Note la gran cantidad de vacuolas (V) y cuerpos residuales en el citoplasma. También se observa unas estructuras similares a poliribosomas (PRB). El contacto de las amibas con otras células o entre ellas es siempre muy íntimo tomando el contorno de la célula vecina (C) (Tomada durante el desarrollo de la tesis).

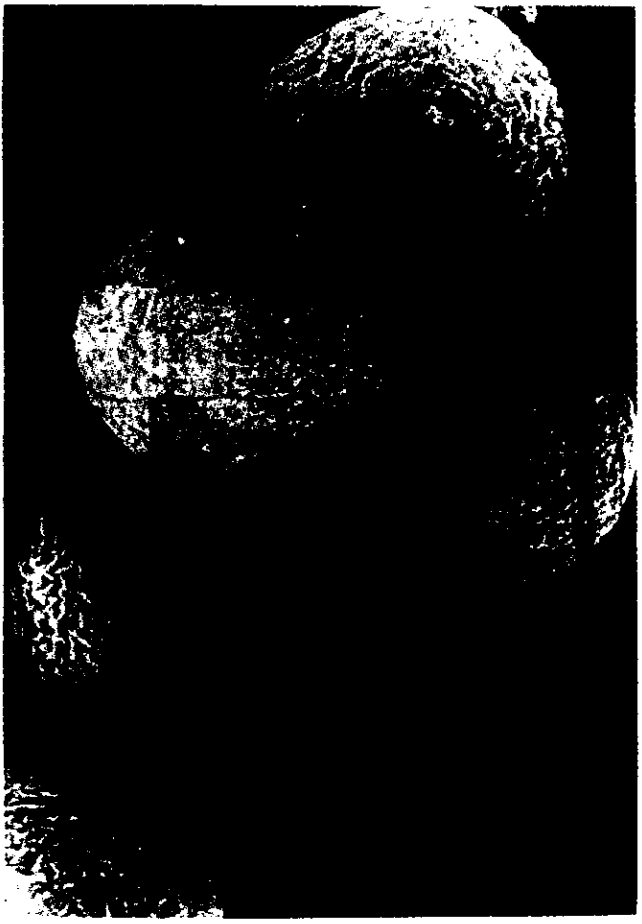


Fig. 3 Apariencia de los quistes de *Entamoeba histolytica* bajo microscopía electrónica de barrido. El quiste es la forma de resistencia de la amiba y propagación de la enfermedad (Martínez-Palomo, 1987).

número exacto de cromosomas. Los ácidos nucleicos en el núcleo de la amiba se distribuyen de manera atípica con respecto al resto de los eucariontes: la cromatina que contiene DNA involucrado en la síntesis del RNA ribosomal se distribuye periféricamente a lo largo del borde interno del núcleo, mientras el DNA involucrado en la síntesis de RNAm se condensa en un endosoma central parecido al nucleolo (Albach, 1989). El tamaño del genoma de la amiba es de 4 x 10^8 bp con aproximadamente 38,400 genes transcripcionalmente activos, lo cual equivale a ocho veces el número de genes activos en *Drosophila melanogaster* (Torres-Guerrero y col., 1991). La mayoría de los genes carecen de intrones y en aquellos en los que se han reportado comprenden sólo unos pocos nucleótidos (Lohia y Samuelson, 1993; Plaimauer y col., 1994). En el genoma de la amiba las regiones codificadoras están asociadas de una manera unidireccional y análisis *in vitro* sugieren que la transcripción se desarrolla de una manera monocistrónica más que policistrónica. Las regiones intergénicas (400-2000 bp) así como las secuencias 5' y 3' no traducibles de los RNAm son relativamente cortas en comparación con el genoma de los eucariotes superiores. De igual forma las secuencias reguladoras del comienzo (ATTCA ó ATCA) y término [TA(A/T)TT] de la transcripción, así como una secuencia tipo caja TATA (TATTTAAA) localizada frecuentemente 30 bp antes del ATG de inicio de la traducción, difieren de las secuencias consenso de los genes de eucariontes (Bruchhaus y col., 1993). Los genes ribosomales residen en más de 200 copias de elementos circulares extracromosomales que miden 25 kb cada uno (Bhattacharya y col., 1989). Cada copia contiene 2 unidades de transcripción de RNAr en posición invertida, que incluyen los genes para las subunidades ribosomales siguiendo el típico arreglo en eucariontes (16s, 5.8s y 25s) (Huber y col., 1989; Sehgal y col., 1994).

Ciclo de vida

El ciclo de vida de la amiba es muy simple. El parásito oscila entre los estadios de trofozoíto (forma invasora) y quiste (forma infectiva) sin estado sexual; el hombre es el único hospedero definitivo y carece de hospederos intermediario y de vectores (Fig. 4).

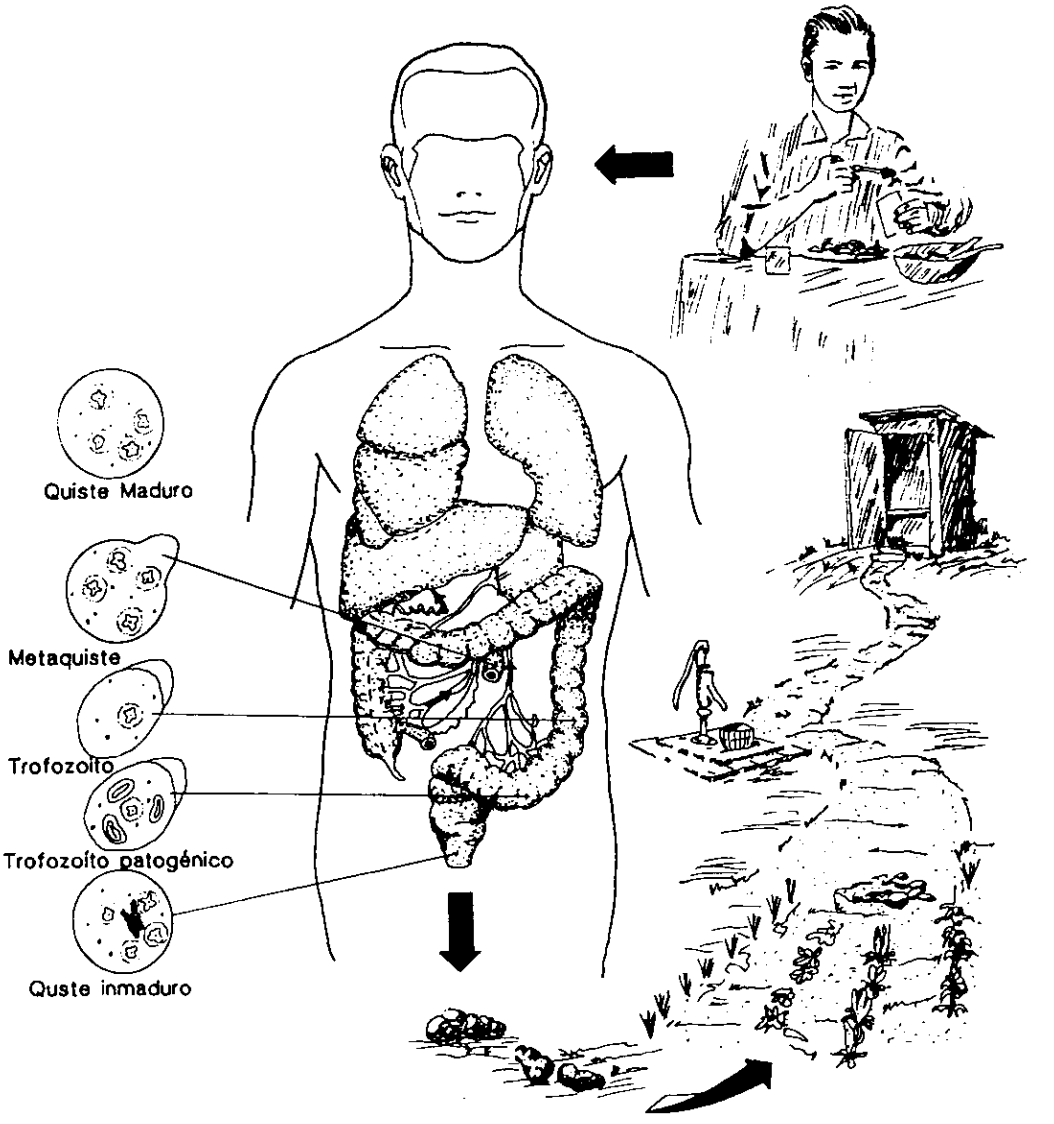


Fig. 4 Ciclo de vida de *Entamoeba histolytica* (Beck y Davies, 1983; Wyler, 1990).

El ciclo se inicia con la ingestión de alimentos y agua contaminados con quistes de *E. histolytica* presentes en materia fecal de individuos infectados o de portadores asintomáticos. Gracias a su cubierta de quitina, los quistes ingeridos resisten a la acción corrosiva de los jugos gástricos hasta alcanzar la parte baja del intestino delgado. A ese nivel los quistes pierden su cubierta de quitina y cada uno de los cuatro núcleos liberados del quiste maduro se divide dando origen a ocho núcleos que se rodean de citoplasma y una membrana celular. El resultado son 8 trofozoítos que se dividen por fisión binaria en la luz intestinal y terminan colonizando el intestino grueso (Barker y Swales, 1972).

En el 10% de la población infectada, los trofozoítos atraviesan las barreras intestinales penetrando en la mucosa, lo que resulta en colitis ulcerativa y disentería amibiana. En el 10% de estos casos los trofozoítos alcanzan las vías mesentéricas y se dirigen al hígado, su principal órgano blanco, causando el denominado absceso hepático amibiano (AHA) responsable de la mayoría de muertes por amibiasis. A través de un proceso aún desconocido, los trofozoítos en la parte baja del intestino grueso sufren un proceso de enquistamiento activo con dos divisiones nucleares, glicólisis anaeróbica y síntesis de DNA (Gonzáles-Robles y Martínez-Palomo, 1983). Los quistes son eliminados con la materia fecal en cantidades cercanas a 45 millones por día (Barker y Swales, 1972). La deposición inadecuada de las excretas y la ausencia de hábitos higiénicos conlleva a la propagación de los quistes cerrando el ciclo de vida.

Mecanismos de patogenicidad

La amiba es una célula con increíble capacidad lítica. Es capaz de destruir casi cualquier tejido que se encuentre a su paso. Aun cuando algunos investigadores sugieren que la amiba es capaz de destruir células a distancia a través de productos que secreta, la mayoría de ellos están de acuerdo en que el efecto citolítico que ejerce la amiba es dependiente del contacto íntimo con la célula blanco (Ravdin y Guerrant, 1981; Ravdin, 1986; Bailey y col., 1990; Saffer y Petri, 1991b). Diversos estudios han demostrado que *E. histolytica* coloniza el intestino grueso del hombre a través de un proceso activo de

adherencia a la mucosa colónica e invasión del epitelio intestinal que lleva a cabo por medio de enzimas, productos tóxicos y moléculas líticas como el amebaporo (Ravdin, 1986; Young y col., 1982; Leippe y col., 1991).

Este proceso combina la habilidad motriz de la amiba para ponerse en contacto íntimo con la célula blanco y la participación de moléculas de superficie que estabilizan la interacción. Ensayos de inhibición de la adherencia con carbohidratos y anticuerpos demostraron que la amiba se adhiere principalmente a través de lectinas y de otras moléculas genéricamente llamadas adhesinas (Ravdin, 1986; Bailey y col., 1990; Saffer y Petri, 1991a; Mann y Petri, 1991). Entre ellas se han reportado dos lectinas, una de 260 kDa (con afinidad por N-acetil-D-galactosamina) y otra de 220 kDa (con afinidad por N-acetil-D-glucosamina); una adhesina de 112 kDa y algunas moléculas inmunogénicas como la proteína de *E. histolytica* rica en serinas (SREHP) (Rosales-Encina y col., 1987; Arroyo y Orozco, 1987; Stanley y col., 1990). De ellas, se ha sugerido que la lectina de 260 kDa, que se trata de un heterodímero constituida por dos subunidades de 170 kDa y 35 kDa unidas por puentes de disulfuro, parece mediar la mayor parte de la adherencia amibiana a la célula blanco. Así, el tratamiento de trofozoítos con N-acetil-D-galactosamina o anticuerpos obtenidos contra la subunidad de 170 kDa (policlonales o monoclonales contra la mayoría de sus epítopes) inhiben la adherencia a células epiteliales en casi el 100% (Ravdin y col., 1989). Sin embargo, estudios realizados por otros investigadores y por nuestro grupo indican que la adherencia amibiana es un proceso complejo en el que participan muchas moléculas de superficie además de dicha lectina (Arroyo y Orozco, 1987; Carrero y col., 1994).

La patogenicidad de la amiba también depende de los niveles de expresión de algunas proteinasas, los cuales en trofozoítos patógenos pueden llegar a ser entre 10 y 100 veces mas altos (Tannich y col., 1991). De igual forma, la alta virulencia de algunos aislados de *E. histolytica* correlaciona con un alto contenido de proteinasas de cisteína o tipo-catepsina en extractos y en productos de secreción de la amiba (Gadasi y Kessler, 1983; Hidalgo y col., 1990). Por otra parte, los trofozoítos contienen dos fosfolipasas A (una dependiente y otra independiente del calcio) que como se ha sugerido hidrolizan diacil-fosfolípidos generando liso-monoacil fosfolípidos que son tóxicos para las membranas celulares (Long-Krug y col.,

1985). La amiba también libera otras enzimas que al parecer participan en la muerte celular y destrucción tisular, como son: la colagenasa, la neuraminidasa, tripsina, pepsina, gelatinasa y enzimas que hidrolizan fibrina, hemoglobina y caseína, entre otras (Ravdin, 1986). Sin embargo, aun se desconocen la serie de eventos que culminan con la destrucción de las células del hospedero después de la adherencia (Bailey y col., 1990).

La respuesta inmune secretoria y la amibiasis.

La inmunoglobulina A presente mayoritariamente en las secreciones externas (IgA de secreción: IgAs), es la primera línea de defensa específica contra las infecciones naturales (Tomasi y Bienenstock, 1968). La IgA de secreción es predominantemente dimérica con dos cadenas polipeptídicas adicionales, unidas por medio de la cadena J ("Join") que desempeña un papel importante en la dimerización de la IgA y por el componente secretor que actúa como receptor específico para el transporte de la IgA dimérica a través de las células epiteliales a los fluídos secretores (Della Corte y Parkhouse, 1973; Brandtzaeg, 1976). La IgA dimérica forma con el componente secretor una macromolécula que adquiere mayor resistencia a la acción de las proteasas, acorde con el micromedio donde ejerce su acción. Esta inmunoglobulina constituye aproximadamente el 90% de los anticuerpos en secreciones (Acosta y Lascurain, 1992).

Los anticuerpos IgAs tienen un amplio espectro de actividades contra virus, bacterias, hongos, parásitos, antígenos del medio ambiente y de los alimentos (Bienenstock y Befus, 1985). La IgAs y mucinas retienen a organismos patógenos dentro de la capa de moco en las mucosas, retardando la invasión y eliminándolos a través de procesos naturales de remoción (Tse y Chadee, 1991). Varios estudios han demostrado que la IgAs tiene la capacidad de inhibir la adherencia y conferir inmunidad contra agentes bacterianos causantes de la placa y caries dental en humanos (Morisaki y col., 1983). Asimismo, se ha descrito que la IgAs es capaz de aglutinar algunos parásitos (Miller, 1987) e incluso conferir

inmunidad en ratones contra el protozoario *Trichuris muris* en ensayos de transferencia pasiva de anticuerpos monoclonales anti-*T. muris* (Roach y col., 1991).

Estudios sobre la respuesta inmune secretoria en la amibiasis y su papel como mecanismo de protección específica son escasos. La mayoría de estos estudios demuestran que la infección intestinal con *E. histolytica* induce una respuesta inmune local en humanos con producción de anticuerpos IgAs específicos anti-amiba que se pueden detectar en secreciones intestinales, leche, calostro y saliva (O'Shea-Alvarez y Treviño, 1985; Grundy y col., 1983; Berber y col., 1990; del Muro y col., 1990). En condiciones experimentales se han inducido anticuerpos IgAs en bilis de ratas inmunizadas con trofozoítos (Acosta y col., 1982), en heces de ratones inmunizados oralmente con trofozoítos fijados con glutaraldehído (Moreno-Fierros y col., 1994; Navarro-García y col., 1997) y en heces de roedores inmunizados por la misma vía con la lectina de 260 kDa con afinidad por N-acetil-D-Galactosamina (Kelsall y Ravdin, 1995) y con una proteína recombinante derivada de SREHP. En el caso de SREHP, se logró una inducción exitosa de la producción de IgAs en roedores con un fragmento de 12 aminoácidos acoplado a la subunidad β de la toxina del cólera (Zhang y Stanley, 1995) así como el mismo fragmento expresado en una cepa atenuada de *Salmonella tiphimurium* que se emplea actualmente en el desarrollo de multi-vacunas (Cieslak y col., 1993). En ambos casos la producción de anticuerpos IgAs anti-SREHP fue escasa; sin embargo, dichos anticuerpos fueron capaces de bloquear la adherencia de trofozoítos a monocapas de células de mamíferos. Recientemente el mismo grupo de trabajo logró expresar en la cepa de *Salmonella* altos niveles de la proteína recombinante mediante el uso de un plásmido que permite obtener un número mucho mayor de copias por célula (Zhang y Stanley Jr, 1996). Un resultado similar obtuvo el grupo de Mann con la subunidad de 170 kDa de la lectina con afinidad por N-acetil-D-Galactosamina (Mann y col., 1997).

A pesar de la inducción de anticuerpos IgAs anti-amiba en animales de experimentación, no existen evidencias directas *in vivo* de que dichos anticuerpos ejerzan un efecto protector contra la infección intestinal. Sin embargo, las observaciones *in vitro* acerca de la capacidad de los anticuerpos IgA de salivas de humanos y de heces de ratones inmunizados con la subunidad de 170 kDa de la lectina de inhibir la adherencia amibiana a

monocapas de células de mamíferos así como los recientes reportes que demuestran que la inmunización oral protege contra la amibiasis hepática, sugieren que los anticuerpos IgAs podrían ejercer algún efecto protector sobre la amibiasis intestinal (Carrero y col., 1994; Kelsall y Ravdin 1995; Zhang y Stanley Jr, 1996; Mann y col., 1997). Tal vez la única evidencia indirecta *in vivo* de que los anticuerpos IgAs podrían tener actividad anti-amiba a nivel intestinal proviene de un reporte que demuestra una baja incidencia de amibiasis intestinal en recién nacidos de un área endémica de Africa durante el período que fueron amamantados por sus madres (Islam y col., 1988). Se sugiere en ese estudio que la protección observada pudo ser debida a la transferencia pasiva de anticuerpos IgAs anti-ameba con la leche materna.

Vacuna oral contra *E. histolytica*.

La idea de una vacuna oral contra la amibiasis no es nueva. Desde hace tiempo, algunos autores vienen sugiriendo que la vacunación oral sería la forma ideal de protección contra patógenos que penetran por vía entérica, como es el caso de la amiba. Además de inducir la activación de los mecanismos inmunes locales, la inmunización oral presenta una serie de ventajas de carácter práctico y económico ya que no se requiere de personal, ni material u equipo especializado para su administración y es ideal para la población infantil debido a que es una técnica no agresiva.

En humanos, la vacunación oral requiere en principio la identificación de antígenos del parásito que estimulen de manera eficaz la inmunidad local (secretora o celular). En el caso de *E. histolytica*, a pesar de que durante la amibiasis intestinal la producción de anticuerpos IgAs está ampliamente documentada (ver arriba), la identidad de las proteínas amibianas que son blanco de esa respuesta es totalmente desconocida. Algunas de estas moléculas son aparentemente equivalentes en peso molecular a algunas proteínas amibianas ya caracterizadas; sin embargo, su verdadera naturaleza aún se desconoce. Evidentemente, una vacuna oral también requiere que la respuesta inmune generada ejerza un efecto

protector sobre la infección parasitaria. En ese sentido, como ya se mencionó, existen algunas observaciones que sugieren un papel protector de los anticuerpos IgAs anti-amiba *in vitro* e *in vivo*; sin embargo no existen evidencias concluyentes del papel biológico de la IgA en esta enfermedad. Recientemente se dio un gran paso en los ensayos iniciales de inmunización oral con antígenos de la amiba, cuando los grupos de Stanley y Mann lograron inducir protección contra el desarrollo de abscesos hepáticos en animales inmunizados por esa vía con proteínas recombinantes de la SREHP y de la lectina con afinidad por galactosa (Zhang y Stanley, 1996; Mann y col., 1997). Como ellos lo sugieren y es el tema que aborda en parte esta tesis, el próximo paso es la evaluación de la capacidad de protección que confiere la inmunización oral contra el desarrollo de la infección intestinal. Para ello, es esencial el desarrollo de un modelo experimental reproducible, confiable y reminiscente de la infección en humanos, que permita evaluar la vacuna oral y definir el papel de los anticuerpos IgAs en el control de la amibiasis intestinal.

Modelos experimentales de amibiasis intestinal.

Los intentos por establecer la enfermedad en modelos animales de experimentación comenzaron desde el mismo momento en que se demostró que la *E. histolytica* es el agente causal de la disentería amibiana y del absceso hepático amibiano en humanos. A pesar de que se ha intentado en muchas especies de animales, hasta hoy no se ha logrado reproducir la infección de manera natural como en el hombre: infección oral con quites, liberación de trofozoítos, colonización del intestino grueso, producción de lesiones y liberación de quistes en las heces. Esto no ha impedido que muchas de esas especies se utilizen para el estudio de lesiones amibianas localizadas, a través de la administración de trofozoítos en el sitio de interés. De esa manera se han desarrollado con éxito algunos modelos experimentales de infección amibiana hepática e intestinal. La introducción de cultivos axénicos de *E. histolytica* (Diamond y col., 1978) ha facilitado el análisis de la infección en los modelos

experimentales, sin embargo la identificación de un modelo ideal se ha visto truncada por nuestra incapacidad para obtener y cultivar quistes en el laboratorio.

En general, las especies animales que se utilizan para el estudio de la amibiasis intestinal experimental son diferentes de aquéllas que se emplean para la amibiasis hepática. La mayoría de los animales en los que se ha intentado establecer una infección amibiana intestinal muestran una serie de limitaciones que hace difícil hacer un seguimiento de la infección, además de que la misma es diferente de la humana en magnitud y duración. Algunas de las especies que se han utilizado en la infección intestinal son: perros, gatos, cobayos, rata, hámster, ratón, conejo y jerbos, entre otros (Tsutsumi, 1988; Meerovicht y Chadee, 1988). Perros y gatos fueron los primeros animales que se emplearon para estudiar la amibiasis intestinal. Ambos son particularmente susceptibles a la infección intestinal con trofozoítos, debido a que no son infectados naturalmente por el parásito y carecen de mecanismos de defensa efectivos contra la amiba. Sin embargo, los estudios en esos animales se abandonaron casi en la mitad del presente siglo, en parte por lo poco práctico y por la extrema susceptibilidad que siempre favorece a la amiba. En los cobayos, a pesar de que se han logrado obtener lesiones cecales y colónicas tanto con trofozoítos monoxénicos como axénicos, los resultados no son reproducibles y el desarrollo de las lesiones dependen de una serie de factores difíciles de controlar.

Las ratas y los conejos son susceptibles pero requieren que los trofozoítos sean administrados en asociación con bacterias, lo que puede enmascarar el efecto real de la amiba en la formación de las lesiones intestinales. El hámster es la especie animal más susceptible a la infección hepática amibiana, sin embargo, desarrollar una infección intestinal es difícil y requiere de trofozoítos asociados a bacterias o altamente virulentos después de varios pases por hígado. En cuanto a los jerbos, son susceptibles a la infección intestinal y a la infección hepática (como el hámster aunque en menor grado). Algunos autores consideran que pueden ser el mejor modelo para el estudio secuencial del desarrollo de lesiones amibianas intestinales y extraintestinales. Así, se ha observado en estos animales que la infección cecal con ausencia de amibiasis invasora conlleva a cambios histológicos en el hígado similares a aquellos que se observan en el humano.

El ratón se ha considerado como una especie naturalmente resistente a la infección por *E. histolytica*. Sin embargo, diversos estudios han demostrado que se pueden inducir lesiones intestinales en ratones dependiendo de la virulencia de la cepa empleada, de si se administra asociada con bacterias y del fondo genético del ratón. Este último punto es particularmente interesante. Ghadirian y Kongshavn (1984), reportaron que la susceptibilidad del ratón a la infección cecal por amibas es genéticamente controlada, demostrando que algunas cepas de ratones eran susceptibles a la vez que otras eran resistentes. El uso del ratón como modelo experimental representa una serie de ventajas desde el punto de vista práctico: su mantenimiento es relativamente económico y sencillo, son de fácil manipulación, su tasa de reproducción es alta y se dispone en el mercado de una amplia gama de productos para la evaluación de cualquier fenómeno de interés.

Gracias al esfuerzo de un grupo de investigadores de nuestro laboratorio, se desarrolló un modelo de amibiasis intracecal en ratones C3H/HeJ que es altamente reproducible, de larga duración y similar a la infección intestinal de humanos (Ghosh y col., 1994). La infección se desarrolló en el 100% de los animales inoculados intracecalmente con trofozoítos cultivados monoxénicamente con *Escherichia coli* y recuperados después de varios pases por hígado. Las típicas lesiones ulcerativas con una ligera disentería amibiana alcanzaron su máximo a los 20 días post-infección y la permanencia de trofozoítos en el ciego se contabilizó hasta 3 meses después. Posteriormente las lesiones tienden a sanar de manera espontánea. A diferencia de este modelo, el desarrollo de las lesiones intracecales en otras cepas de ratones es de muy corta duración (Rivero-Nava y col., 1997) como para realizar un seguimiento de la infección y evaluar el papel de la propia respuesta inmune del hospedero o de una respuesta inmune inducida con antígenos del parásito sobre el desarrollo de la amibiasis intestinal. Recientemente nosotros hemos reproducido el modelo de amibasis cecal descrito por Ghosh y col., usando trofozoítos axénicos altamente virulentos después de varios pases por hígado de hámster, lo cual facilita la interpretación de resultados derivados del uso de este modelo experimental, que representa una herramienta muy útil en el estudio de la infección intestinal por *E. histolytica*, así como en la evaluación de vacunas potenciales contra la amibiasis intestinal.

Planteamiento del problema

A excepción de la lectina con afinidad por galactosa, se desconoce la identidad de las proteínas de *E. histolytica* que son reconocidas por los anticuerpos IgAs humanos y que como tal podrían actuar como inmunógenos orales en el mismo. En consecuencia, asociado al problema de la falta de un modelo experimental de amibiasis intestinal, no existen estudios de protección contra la misma. La administración oral de proteínas de *E. histolytica* en el humano y en particular aquéllas que son inmunogénicas al sistema inmune de las mucosas, podría generar una efectiva respuesta local con producción de anticuerpos IgAs específicos que impidan, probablemente a través del bloqueo de la adherencia amibiana, la colonización del intestino grueso por la amiba y consecuentemente el desarrollo de la enfermedad.

En el presente trabajo se decidió determinar por técnicas de biología molecular la identidad de algunas de las proteínas de *E. histolytica* relevantes a la respuesta inmune secretoria en pacientes y portadores asintomáticos, así como la capacidad por medio de inmunización con proteínas recombinantes derivadas de algunas de los antígenos identificados, de proteger a una cepa de ratones susceptible al desarrollo de amibiasis intestinal.

OBJETIVO

Clonar antígenos de *E. histolytica* potencialmente inductores de respuesta inmune secretoria en humanos y evaluar *in vivo*, en un modelo murino, el papel de los anticuerpos IgAs como posible mecanismo protector contra la amibiasis intestinal invasora.

DESARROLLO EXPERIMENTAL

-. Clonar a partir de una librería de cDNA de *E. histolytica* los genes de antígenos reconocidos predominantemente por la respuesta inmune secretoria (IgAs) de personas infectadas (salivas de enfermos y portador asintomático).

-. Secuenciar total o parcialmente la mayoría de las clonas aisladas y determinar su naturaleza o identidad.

-. Caracterizar molecular y bioquímicamente algunos de los antígenos identificados.

-. Hacer un estudio comparativo de los antígenos identificados a través de la clonación con las salivas de personas enfermas (pacientes con amibiasis intestinal y absceso hepático amibiano) y con la de un portador asintomático.

-. Expresar algunos de los antígenos seleccionados como proteínas de fusión.

-. Inducir respuesta inmune secretoria en ratones C3H/HeJ por inmunización oral con las proteínas recombinantes.

-. Evaluar la capacidad de los anticuerpos IgAs, inducidos por inmunización oral de los ratones, de conferir protección contra la infección amibiana intracecal.

MATERIALES Y MÉTODOS

Cultivo y cosechamiento de E. histolytica. Trofozoítos axénicos de *E. histolytica*, cepa HM-1:IMSS, se crecieron en medio de cultivo TYI-S-33 (Diamond y col., 1978) complementado con 15% de suero bovino adulto, 100 U/ml de penicilina y 100 µg/ml de sulfato de estreptomicina a 37°C. Los trofozoítos se cosecharon a las 72 h de cultivo (fase de crecimiento logarítmica) por enfriamiento en hielo durante 10 min y centrifugación a 150 x *g* durante 5 min a 4°C. Para la obtención de extracto total, los trofozoítos de *E. histolytica* lavados con PBS, pH 7.4, se resuspendieron en amortiguador de Tris-HCl 50 mM, pH 7.5, con inhibidores de proteasas (PHMB y PMSF) y se lisaron por 5 ciclos continuos de calentamiento y enfriamiento en hielo seco. Un extracto citosólico se obtuvo a partir del lisado total por centrifugación a 60,000 x *g* durante 30 min a 4°C, recuperando el sobrenadante al cual se le hizo determinación de proteínas por el método de Lowry (Lowry y col., 1951) y se almacenó en alícuotas a -20°C hasta su uso.

Muestras de saliva. Se obtuvieron muestras de saliva de pacientes con amibiasis intestinal y de un paciente con AHA en la unidad de coloproctología del Hospital General de México con la generosa ayuda del Dr. Edmundo Godínez Camacho. El diagnóstico de la amibiasis intestinal se realizó por análisis coproparasitoscópico, rectosigmoidoscopía y detección en saliva de anticuerpos IgA anti-amiba por ELISA (Del Muro y col., 1990). El AHA se diagnosticó por ultrasonido y observación microscópica de trofozoítos de amiba en muestras de biopsia hepática. También se obtuvo muestra de saliva de un individuo portador asintomático de quistes de *E. histolytica* y con anticuerpos IgA anti-amiba en saliva. Todas las muestras de saliva se colocaron en hielo inmediatamente después de ser colectadas y se centrifugaron a 10,000 x *g* durante 30 min a 4°C para limpiarlas de *detritus* y moco. Los sobrenadantes se separaron en alícuotas y congelaron a -20°C hasta su uso.

Bibliotecas de cDNA y genómica de E. histolytica. La bibliotecas de DNA complementario (cDNA) y de DNA genómico (gDNA) de *E. histolytica*, cepa HM1-IMSS, que se utilizaron en el presente trabajo fueron proporcionadas por la Dra. Esther Orozco del Centro de Investigación y Estudios Avanzados del IPN y el Dr. Abraham Landa de la Facultad de Medicina de la UNAM. Ambas bibliotecas se prepararon en el vector λ ZAPII utilizando un estuche (Uni-ZAP™ XR Cloning Kit) distribuido por la casa comercial Stratagene, USA. Este estuche incluye todo lo necesario para la síntesis del cDNA, la clonación unidireccional en el bacteriofago λ ZAPII (biblioteca de expresión) y el empaque del mismo. Para la biblioteca de cDNA, se obtuvo RNAm de *E. histolytica* cepa HM1-IMSS por los métodos convencionales y el cDNA preparado a partir del mismo se clonó entre los sitios EcoRI (5´) y XhoI (3´) de λ ZAPII (Fig. 5). En cuanto a la biblioteca genómica se utilizó DNA total de *E. histolytica* cortado con la enzima EcoRI.

Tamizaje de la biblioteca de cDNA con las muestras de saliva. Se realizó un inmuno-tamizaje sobre réplicas de más de 10^5 placas líticas de la biblioteca de cDNA, con cada tipo de muestras de saliva pre-absorbidas extensamente con lisado de *Escherichia coli* para remover las especificidades no deseadas (Carrero y col., 1997). Aproximadamente 10^5 fagos de la biblioteca de cDNA se incubaron con 200 µl de un cultivo de toda la noche de bacterias XL1-Blue, resuspendido en 5ml de $MgSO_4$ 10 mM por 15 min a 37 °C, se mezcló con el agar superior y se vació sobre cajas de Petri con agar de fondo. Las cajas de Petri se incubaron toda la noche a 37 °C hasta la aparición de las placas líticas. Filtros de nitrocelulosa empapados con 10 mM de isopropyl-thio-β-D- galactopyranoside (IPTG) se incubaron toda la noche a 37 °C sobre la biblioteca plaqueada en las cajas de Petri. Al día siguiente, los filtros-réplicas se bloquearon con BSA 3% en amortiguador TNT (Tris-HCl 10 mM, pH 8.0; NaCl 150 mM; Tween 20, 0.05%) por 1 h a 37 °C y se incubaron por separado durante toda la noche a 4 °C con una dilución 1:2 de los tres tipos de salivas absorbidas (mezcla de salivas de pacientes con amibiasis intestinal, saliva de un paciente con AHA y saliva de un sujeto portador asintomático) en amortiguador de bloqueo. Los filtros-

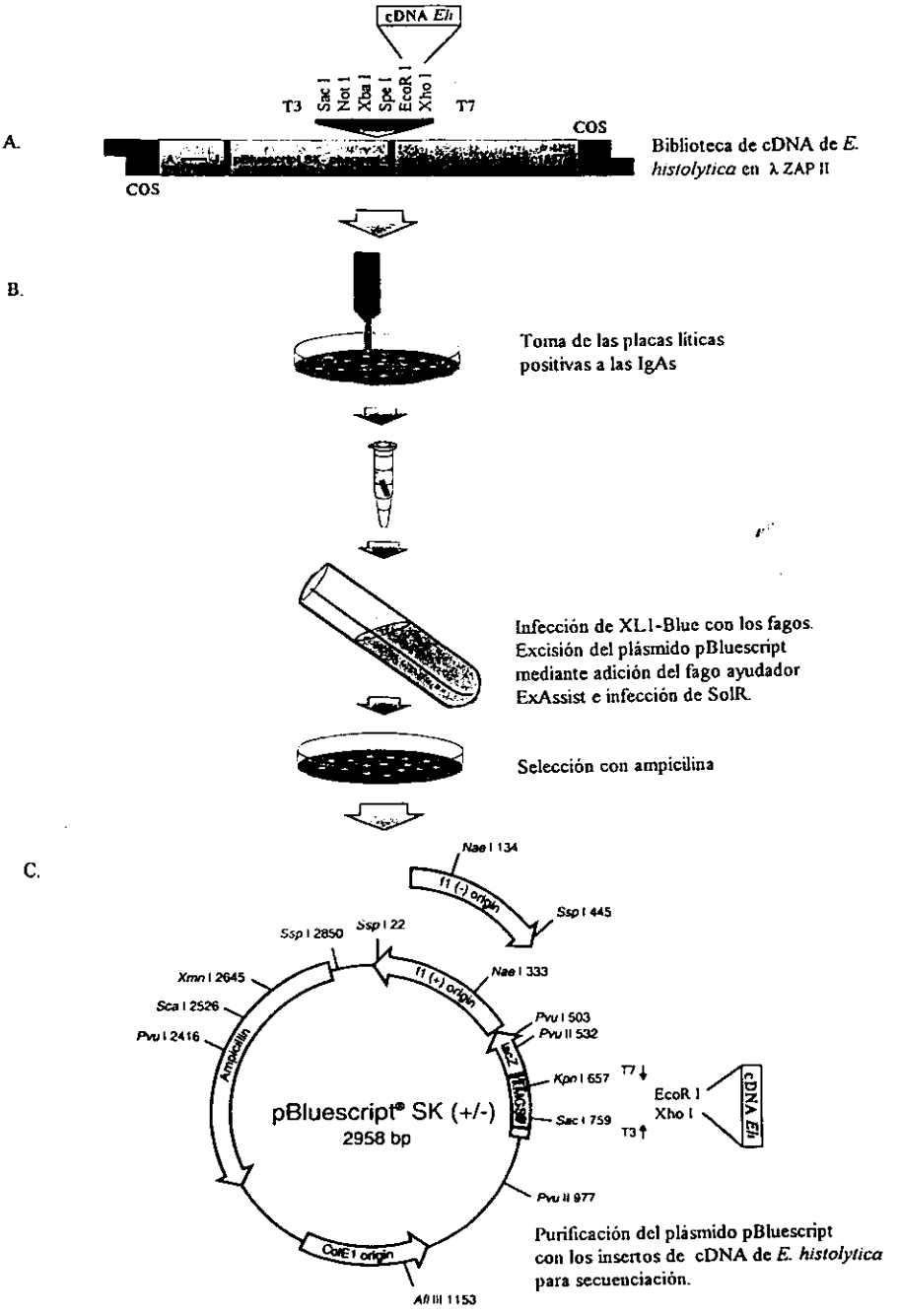


Fig. 5 Clonando genes de la amiba en el vector λ ZAPII. Este tipo de vectores denominados fagémidos combina en una las ventajas que ofrece por separado trabajar con bacteriófagos y plásmidos (A). Las clonas aisladas que contiene el inserto de interés (indicado en un recuadro) en el sitio de multiclonación MCS, son sometidas a un proceso sencillo de excisión (B) que da origen al plásmido pBluescript SK (C) de donde serán secuenciadas.

replicas se lavaron 3 veces con BSA-TNT 1%, Nonidet P-40 0.1% y se incubaron con un anticuerpo de cabra anti-IgA humano acoplado a peroxidasa (diluido 1:1000 en BSA-TNT, 3%), durante 2 h a temperatura ambiente. Después de tres lavados igual que los anteriores, los complejos inmunes en los filtros-replicas, se revelaron usando 4-chloro-1-naphtol como substrato. Las placas líticas positivas se picaron de la caja de petri original, se suspendieron en amortiguador SM (MgSO4 10 mM, 0.01% de gelatina en Tris 50 mM, pH 7.5) con 2% de cloroformo y se guardaron a 4°C hasta su uso. Las placas líticas positivas a cada una de las muestras de saliva, se aislaron totalmente después de tamizajes secundarios y terciarios.

Excisión de fagémidos. El vector λ ZAPII tiene la particularidad de que se puede manipular para dar origen a un plásmido (fagémido pBluescript SK(-); Fig. 5), que contiene los sitios de multiclonación y por ende los insertos, a través de un proceso de excisión que necesita de un fago ayudador y de una cepa bacteriana de *E. coli* no supresora, que impide la replicación del fago ayudador. Los fagos de las placas positivas a las muestras de salivas, se sometieron al proceso de excisión, usando el sistema del fago ayudador ExAssist y la cepa bacteriana SOLR de Stratagene (USA) de acuerdo a las instrucciones del fabricante.

Secuenciación de DNA y análisis computacional. La secuenciación de los insertos de DNA de *E. histolytica* se realizó sobre los fagémidos excindidos mediante el método de terminación de cadenas con dideoxynucleótidos (Sanger y col., 1977), usando el sistema de Sequenase 2.0 (United State Biochemical Corporation) y [α-^{35}S]dATP (Amersham International plc). Los fagémidos se purificaron a partir de cultivos de toda la noche de las bacterias SolR transformadas, usando el estuche para purificación de plásmidos de Qiagen (USA). El DNA de doble cadena se desnaturalizó mediante hidrólisis alcalina y se precipitó con etanol, dando lugar a DNA de cadena sencilla que se utilizó como molde en la reacción de secuencia. Se utilizaron los "primers" universales del fago M13 (-20, -40 y reverse) proveídos con el estuche y que se alinean a ambos lados del sitio de multiclonación (Fig. 5). En algunos casos para completar la secuencia, se utilizaron oligonucleótidos sintéticos

diseñados para que se alinearan a sitios internos dentro de los insertos. La secuencia de aminoácidos deducida (marcos de lectura abiertos), así como la determinación de algunas de sus características físico-químicas (hidrofilicidad, hidrofobicidad, conformación espacial, etc.) se realizó con ayuda del programa de análisis molecular PC/Gene de Intelligenetics, Inc. De igual forma, las secuencias de nucleótidos y de aminoácidos deducidas de las clonas de cDNA, se analizaron para determinar secuencias homólogas en la base de datos del National Center of Biotechnology and Ingeniery (NCBI), usando el servicio de alineamiento y análisis de homología BLAST.

Elaboración de las construcciones de expresión y expresión de antígenos recombinantes de E. histolytica. La expresión de la región codificadora de un par de clonas de cDNA seleccionadas (Zsp1 que codifica para la proteína rica en cisteínas de 29 kDa, CREHP y SalJC1 que codifica para EHSalJC1), se realizó en bacterias *E. coli* por subclonación en el vector de expresión pRSET$_B$ (InVitrogen Corp.) (Fig. 6A). Los insertos completos de ambas clonas de cDNA se excindieron de sus respectivos fagémidos con las enzimas de restricción BamHI-XhoI, se purificaron a partir de geles de agarosa y se ligaron al vector pRSET-B cortado con las mismas enzimas. La modalidad B del vector pRSET permitió que los insertos de las clonas de cDNA ligados a esos sitios de restricción quedaran en marco con el ATG del fragmento del gen de la β-galactosidasa (Fig. 6B). La reacción de ligación se llevó a cabo toda una noche a 14 °C en un baño de agua, empleando DNA ligasa de fago T4. Con estas construcciones (pRSET$_B$-Zsp1 y pREST$_B$-SalJC1) se transformaron bacterias de la cepa XL1-Blue por electroporación y se seleccionaron las colonias de bacterias transformadas por crecimiento en presencia de ampicilina (100 μg/ml), a la cual confiere resistencia el plásmido. Las ligaciones en marco de los insertos de cDNA fueron confirmadas por secuenciación de los plásmidos recombinantes, usando oligonucleótidos diseñados para que se alinearan a ambos lados del sitio de multiclonación de pRSET. Las construcciones de expresión contienen además el fragmento de secuencia BamHI-EcoRI del sitio de multiclonación del fagémido pBluescript SK, así como la secuencia del adaptador "GGCACGAG" que precede a las secuencias de los cDNA clonados. Para la expresión de

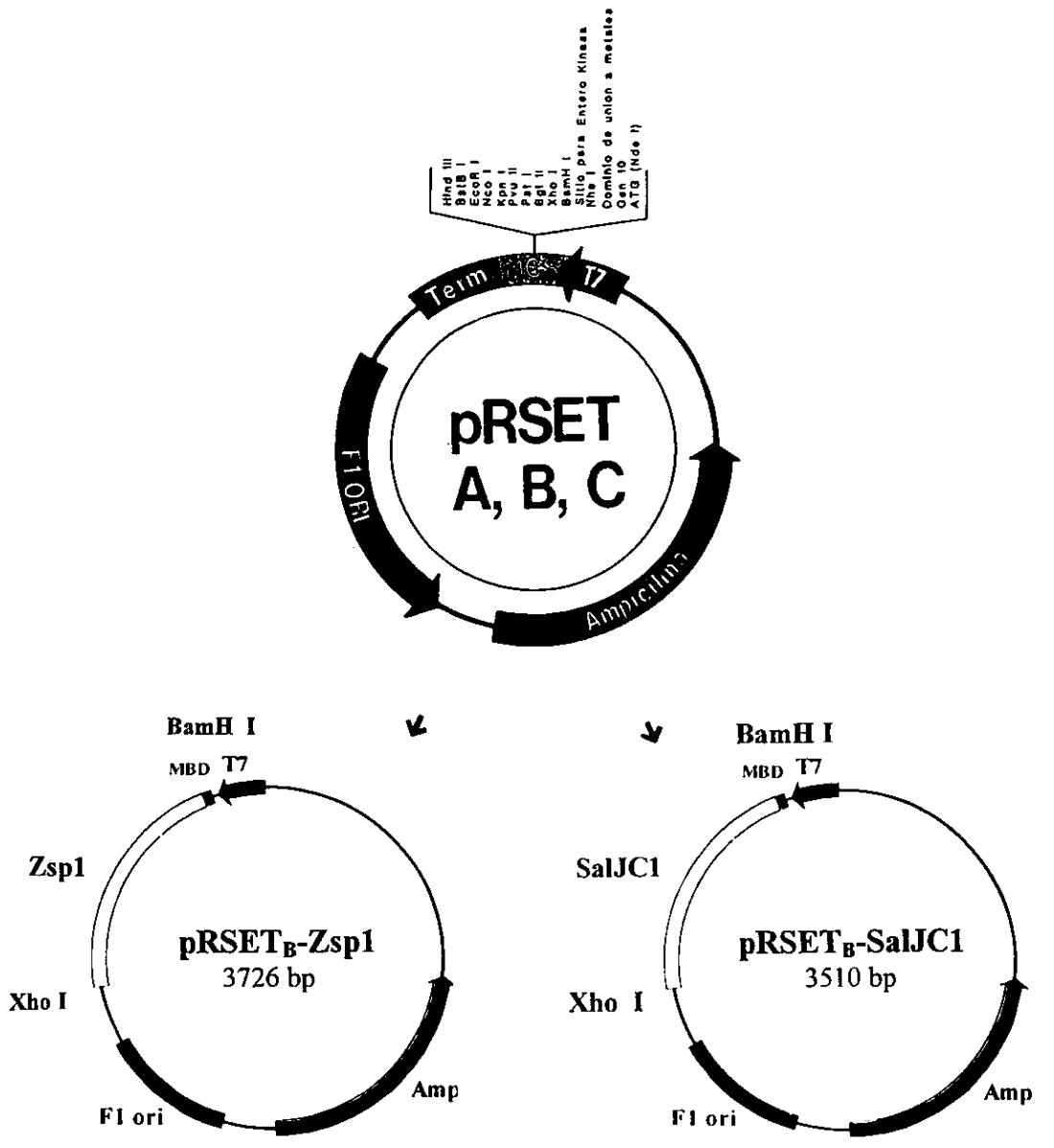


Fig. 6A Representación esquemática del vector de expresión procariótico pRSET y de las construcciones para la expresión de dos proteínas clonadas de *E. histolytica*. Los insertos de las clonas Zsp1 y SalJC1 fueron subclonados entre los sitios BamHI y XhoI del sitio de multiclonación (MSC) del vector, generando las construcciones pRSET$_B$-Zsp1 y pRSET$_B$-SalJC1. Las proteínas recombinantes expresadas contienen un tallo de poli-histidinas que permite la purificación de las mismas por cromatografía de afinidad a metales.

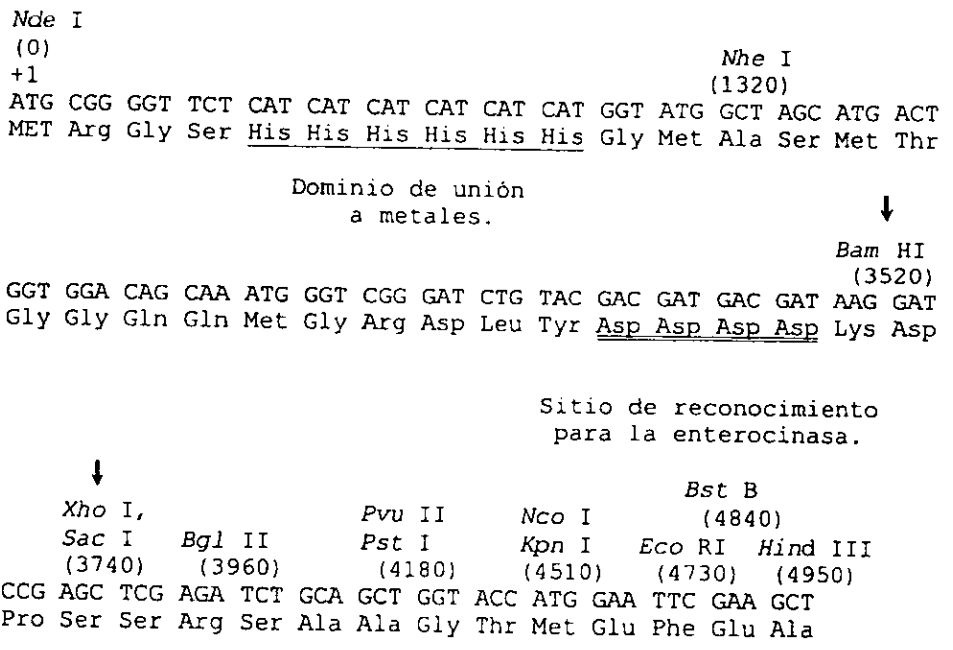


Fig. 6B Secuencia de nucleótidos y aminoácidos del fragmento del vector pRSET$_B$ que comprende desde el codón de inicio de la transcripción del promotor T7 hasta el final del sitio de multiclonación. Los insertos completos de las clonas Zsp1 y SalJC1 se subclonaron entre los sitios BamH I y Xho I, señalados por las flechas. La secuencia que codifica para el tallo de 6 poli-histidinas (dominio de unión a metales) está subrayada, mientras que el sitio de reconocimiento para la enterocinasa se indica por un doble subrayado.

las proteínas recombinantes, cultivos de toda la noche de las bacterias transformadas se diluyeron 1:50 en medio de cultivo SOB con $MgSO_4$ 10 mM conteniendo 100 μg/ml de ampicilina y se crecieron a 37 °C en agitación vigorosa hasta obtener una OD_{600}=0.3. La expresión de peptidos de fusión se indujo con la adición al medio de IPTG 2mM y una hora después el fago M13-T7. Para determinar el tiempo óptimo de expresión, se tomaron muestras de cultivo a intervalos de 1h durante 5h, se centrifugaron y la pastilla de bacterias se mezcló con amortiguador de corrida y se separó por electroforesis en geles de poliacrilamida con SDS (SDS/PAGE) al 12%. De igual forma se determinó la localización celular de los péptidos de fusión por sonicación de la pastilla de bacterias en amortiguador de sonicación (NaCl 300 mM en Na-fosfatos 50 mM, pH 7.8) y centrifugación a 10,000 x *g* durante 20 min (localización citosólica).

Purificación de antígenos recombinantes. La purificación de las proteínas de fusión obtenidas en el vector pRSET se basa en la codificación por parte del plásmido en el extremo amino-terminal de la proteína, de un fragmento de 6 histidinas continuas que confieren afinidad a cationes metálicos. Los péptidos de fusión de *E. histolytica* (CREHP y EHSalJC1) se purificaron por cromatografía de afinidad de unión a metales, usando columnas quelantes Hi Trap (Pharmacia Biotech, USA.) tratadas con cloruro de níquel. La fracción soluble de las bacterias transformadas e inducidas, se aplicó lentamente a las columnas tratadas con níquel, se lavó y las proteínas unidas se eluyeron con EDTA 0.05 M, pH 7.2. La pureza de los péptidos de fusión se determinó por SDS/PAGE al 12%. En todos los casos, se determinó la concentración de proteína por el método de Lowry, usando BSA como estándar (Lowry y col., 1951).

Análisis de Northern blot. Se purificó RNAm poli-A^+ a partir de 10^8 trofozoítos *de E. histolytica*, cepa HM1:IMSS, por extracción con isotiocianato de guanidina y cromatografía en columnas de celulosa-oligo(dT), usando el estuche para purificación de RNAm: QuickPrep Micro mRNA purification kit (Pharmacia Biotech, USA). El RNAm se corrió por

electroforesis en geles de agarosa al 1.2% con formaldehído 2.2M y se transfirió a membranas de nylon Hybond-N (Amersham International, plc), empleando el sistema de vacio Vacu-Gene XL blotting (Pharmacia Biotech, USA). Las tiras de papel transferidas se trataron con una solución de bloqueo (Boehringer Mannheim, Alemania) durante 2 h a 60 °C y se hibridaron con las sondas de cDNA radiomarcadas durante toda la noche a 60°C, en condiciones de mediana astringencia: 0.5 x SSC/0.1% SDS. Al día siguiente los filtros se secaron y se expusieron a una película de impresión de rayos X (Kodak film XR) durante toda una noche a 4 °C. Se utilizaron como sondas los insertos de las clonas de cDNA seleccionadas (Saha51, Saha54 y SalJC1), purificados a partir de geles de agarosa y radiomarcados con [^{32}P] dCTP, empleando el sistema de marcaje de DNA, RTS RadPrime de Life Technologies, USA.

Ratones. Ratones machos de 8 semanas de la cepa C3H/HeJ (Jackson Laboratory. Bar Harbor, Maine USA.) se mantuvieron en jaulas de plástico estériles con filtro de aire y con alimento y agua *ad libitum*. Todos los ratones estaban libres de parásitos, como se determinó en análisis coproparasitoscópico seriado. Veinte ratones se dividieron en 4 grupos de 5 ratones cada uno. Dos grupos (I y II) se inmunizaron con los péptidos recombinantes de las CREHP y EHSalJC1 respectivamente, más toxina del cólera (TC) como adyuvante y posteriormente se infectaron intracecalmente con trofozoítos de *E. histolytica* (como se indica posteriormente). El grupo III sólo recibió la TC y se infectaron, mientras el grupo IV se utilizó como control sano (no imunizados, no infectados).

Inmunización de ratones con los péptidos recombinantes. Los ratones del grupo I y II bajo ligera anestesia con éter, se inmunizaron intragástricamente con una sonda de plástico (Standard wall spaghetti tubing. Chemplast Inc. USA) los días 0, 7 y 21. Cada dosis contenía 250 µg de los péptidos de fusión CREHP (grupo I) y EHSalJC1 (grupo II) con 10 µg de TC (Sigma) como adyuvante en 0.25 ml de una solución de $NaHCO_3$ 0.2M, pH 8.3. Se dio además un refuerzo intraperitoneal el día 14 con 25 µg de los péptidos de fusión,

emulsificados en adyuvante incompleto de Freund (Gibco Laboratories). Los ratones del grupo III sólo recibieron amortiguador de bicarbonato con TC por vía intragástrica y PBS con adyuvante incompleto de Freund por vía intraperitoneal. Al día 25 después del inicio del protocolo de inmunización, se obtuvieron muestras de heces y sueros de los ratones para la determinación de anticuerpos IgA en las heces y anticuerpos séricos totales anti-*E. histolytica* por la técnica de ELISA. Brevemente, en el caso de las heces, el equivalente a aproximadamente 0.1 g de materia fecal se disgregó en amortiguador de fosfatos por agitación en un vórtex y se centrifugó a 10,000 x g durante 10 min. El sobrenadante sin diluir se utilizó inmediatamente en ensayos de ELISA para la cuantificación de IgAs y alícuotas del mismo se congelaron a -20°C para repetir los ensayos (De Vos y Dick, 1991).

Ensayos de ELISA. Placas de 96 pozos se sensibilizaron con 10 µg/pozo de un extracto total de trofozoítos en amortiguador de carbonatos pH 9.6 durante toda la noche a 4°C; se bloquearon con pBS-BSA al 1% durante 1h a 37°C y se incubaron con los sobrenadantes de las heces (sin diluir) y con los sueros de los ratones (diluídos 1:500 en 1% BSA-PBS) durante toda una noche a 4°C. Como segundo anticuerpo, en el caso de los sobrenadantes de heces, se añadió un anticuerpo obtenido en cabra anti-IgA de ratón conjugado a peroxidasa (diluido 1:1000; Zymed Laboratories, Inc.) durante 2 h a 37°C. En el caso de los sueros se empleó anticuerpos obtenidos en cabra anti-globulinas totales de ratón (IgG, IgM e IgA) y contra cada uno de los isotipos por separado, igualmente conjugados a peroxidasa y a la misma dilución (Zymed Laboratories, Inc.). Finalmente, la placa se reveló con orto-fenilenediamina y se leyó en un lector de ELISA a 490 nM. Cada muestra fecal y de suero se probó por duplicado.

Ensayos de inmunotransferencia. Para la inmunotransferencia, una concentración de proteína de 20 ug/carril del extracto total de trofozoítos (ver sección de cultivo del parásito) se corrió en SDS/PAGE al 10 % y se transfirió a papel de nitrocelulosa usando métodos estándar (Towbin y col., 1979). Tiras de 3 mm de ancho se bloquearon durante 1 h a 37°C

con BSA 1% en PBS y se incubaron toda la noche con la mezcla de los sueros de ratones diluídos 1:500 en PBS-BSA. Al día siguiente se lavaron 3 veces con PBS-Tween 0.3%-BSA 1% y se incubaron 2 h a temperatura ambiente con un anticuerpo obtenido en cabra anti-IgG de ratón conjugado a peroxidasa (diluido 1:1000; Zymed Laboratories, Inc.). Después de lavar igual que antes, los conjugados antígeno-anticuerpo finalmente se revelaron con O-cloro naftol.

Infección intracecal de los ratones con trofozoítos de E. histolytica y análisis histopatológico de los ciegos. Al día 28 después del inicio del protocolo de inmunización, los ratones inmunizados (grupos I y II) así como el grupo III se infectaron intracecalmente con 5 x 10^5 trofozoítos axénicos de *E. histolytica*, recuperados después de varios pases por hígado de hámster (AHA) lo cual incrementa su virulencia (como se indica posteriormente). Para la infección, los ratones se anestesiaron con Ketamina (90 mg/kg de peso) y Xilazina (10 mg/kg de peso); se afeitó la área abdominal y se practicó una pequeña laparatomía bajo condiciones asépticas para exponer el ciego. Los trofozoítos, lavados y resuspendidos en 0.1 ml de PBS se inocularon directamente hacia el ápice del ciego, se bloqueó el orificio de la inoculación con algodón y cuidadosamente se regresó el ciego a la cavidad abdominal. La pared abdominal como la piel se cerraron con sutura quirúrgica (Vyclil 4-0) y se aplicó localmente peróxido de hidrógeno al 2% como desinfectante. Los ratones se sacrificaron el día 20 post-infección, ya que es el tiempo en el cual se observan las lesiones más extensas reportadas para este modelo murino de amibiasis intestinal (Ghosh y col., 1994). El día de sacrificio (día 48 desde el inicio de la inmunización) se colectaron muestras de heces y suero de los ratones para la determinación de anticuerpos de secreción y séricos anti-*E. histolytica* por ELISA, como se describió anteriormente. Los ciegos de los animales se removieron y se evaluaron macroscópicamente en cuanto a tamaño, apariencia, color e irrigación y el contenido cecal en cuanto a consistencia, color, mucosidad, rastros de sangre y presencia de trofozoítos por microscopía de luz. Posteriormente se fijaron en formalina al 10%, pH 7.2 y se embebieron en parafina. Para el análisis histopatológico, secciones del tejido teñidas con hematoxilina-eosina se examinaron y fotografiaron bajo microscopía de luz.

Hámsters e inducción de AHA. Hámsters dorados machos de 6 a 8 semanas de edad se mantuvieron en jaulas de plástico estériles con alimento y agua *ad libitum*. Los abscesos hepáticos, tanto para aumentar la virulencia de la amiba así como para los ensayos con la ciclosporina A (CsA, ver adelante), se indujeron por inoculación directa de 10^5 trofozoítos en la vena portal seguido del bloqueo del sitio de inoculación con almohadillas de gel foam. La pared abdominal y la piel se cerraron con sutura quirúrgica (Viclyl 4-0) y la herida se desinfectó con peróxido de hidrógeno al 2%. Los animales se sacrificaron 7-15 días después y fragmentos de hígado con abscesos se incubaron en medio TYI-S-33 con antibióticos para recuperar las amibas.

Actividad de peptidilprolil cis-trans isomerasa (PPIase). La actividad de PPIasa se determinó en la fracción citosólica de trofozoítos según el método descrito por Fisher y col. (1984). El método consiste en la cuantificación espectrofotométrica de la hidrólisis de la quimiotripsina causada por la isomerización *cis-trans* del péptido *N*-succinil-Ala-Ala-Pro-Phe-*p*-nitroanilida. La reacción se realizó en amortiguador Tris-HCl 100 mM, pH 7.5, con 50 µg/ml de quimiotripsina (diluida en el amortiguador de reacción) y 60 µM del péptido (stock de 2 mM en DMSO) y se inició con la adición de 15 µl del extracto citosólico de amibas a una concentración final de 17.8 mg/ml. El curso de la reacción se midió de manera continua a 390 nM durante 150 seg a 4°C. Para los ensayos de inhibición por CSA, se añadieron diferentes concentraciones de la droga (0.02, 0.2, 2, 20 y 50 µg/ml) disuelta en etanol a la mezcla de reacción, ajustando el volumen de etanol a un porcentaje no mayor de 1.5% del volumen total

Tratamiento de trofozoítos de E. histolytica con Ciclosporina A (CsA). Cultivos de trofozoítos de *E. histolytica* cepa HM1:IMSS de 24 h de crecimiento (2.5×10^5), se trataron con CsA a concentraciones de 10^{-3}, 10^{-2}, 10^{-1}, 1, 10 y 50 µg/ml por 48 h a 37°C. Debido a su carácter liposoluble, la CSA se preparó en etanol y se guardó a -20°C hasta su uso. El

volumen de etanol que se añadió con la CSA a cada cultivo fue equivalente a 1:1000 del volumen total. Como control se usó un cultivo con etanol sin CsA. La proliferación y viabilidad de los trofozoítos tratados se determinó a las 72 h de cultivo por conteo en cámara de Neubauer y exclusión de azul de tripano, respectivamente. Cada dosis se probó por duplicado en tres ensayos independientes.

Ttratamiento de hámsters con CSA e inducción de AHA. Tres grupos de 8 hámsters de 6 a 8 semanas de edad y con un peso promedio de 100 g fueron tratados con concentraciones de 3.5, 7.0 y 15.0 mg/kg de CsA en aceite de olivo (Sandoz). Un grupo control con el mismo número de hámsters recibió solo aceite de olivo. La droga se administró intraperitonealmente a las concentraciones indicadas en 200 µl de volumen total, los días 0, 1 y 2 posteriores a la infección hepática de los hámsters con trofozoítos de *E. histolytica*. En breve, los hámsters se anestesiaron con Ketamina y se practicó una laparotomía en condiciones asépticas. Los trofozoítos (1×10^5) con alta virulencia después de varios pases por hígado de hámster, se inocularon directamente en la vena portal y el orificio de inoculación se bloqueó inmediatamente con almohadillas de gel foam. La pared abdominal y la piel se cerraron con sutura quirúrgica (Viclyl 4-0) y la herida se desinfectó con peróxido de hidrógeno al 2%. Los animales se sacrificaron una semana después (día 7) con exceso de anestesia y se analizó el grado de desarrollo de los abscesos hepáticos.

Nota.

Las secuencias de EHSaIJC1 y de la ciclofilina de *E. histolytica* (EhCyP) se encuentran disponibles en la base de datos del GeneBank con los números de acceso U66671 y AF017993, respectivamente.

RESULTADOS

Con la finalidad de identificar antígenos de *E. histolytica* relevantes en la respuesta inmune secretoria, se realizó un inmuno-tamizaje de una librería de cDNA con anticuerpos IgA de saliva de pacientes con amibiasis intestinal, de un paciente con AHA y de un portador asintomático. Se aislaron un total de 18 clonas de las cuales 10 se caracterizaron total o parcialmente (Tabla I).

Clonas de cDNA aisladas con saliva de pacientes con amibiasis intestinal (clonas Zsp). Se aislaron 8 clonas de las cuales se caracterizaron 4. Dos de las clonas denominadas Zsp6 y Zsp7 mostraron insertos idénticos de 555 bp con dos marcos de lectura abiertos (ORF) que se traslapan y que codifican para péptidos de 170 y 166 aminoácidos (Fig. 7A). La investigación en la base de datos mostró que Zsp6 y Zsp7 muestran completa homología con la secuencia reportada para la proteína rica en serinas de *E. histolytica* (SREHP, Stanley y col., 1990). La SREHP es una proteína de 25 kDa, muy inmunogénica, que muestra entre otras repeticiones en el extremo carboxilo-terminal, una serie de 5 dodecapéptidos seguido de 4 octapéptidos en tandem. A pesar de que las clonas Zsp6 y Zsp7 son incompletas, la secuencia muestra 48 bp adicionales en la posición 581 con respecto a la clona c1 (clona de la SREHP reportada) que codifican para 2 octapéptidos en tandem en la región de secuencias repetidas del extremo carboxilo-terminal. Como consecuencia, a diferencia de SREHP, las clonas Zsp6 y Zsp7 muestran 2 octapéptidos adicionales formando una proteína con 6 octapéptidos en lugar de 4 (Fig 7B y C). El otro ORF que se traslapa codifica para una proteína rica en ácido glutámico que aparentemente no se expresa en trofozoítos. Otra clona denominada Zsp1 mostró un inserto de 717 bp que contiene un ORF de 227 aminoácidos idéntico al de la proteína de 29 kDa rica en cisteínas de *E. histolytica* (Torian y col., 1990). La secuencia de Zsp1 carece de los primeros 7 aminoácidos del extremo amino-terminal y sólo muestra con respecto a la secuencia previamente reportada, un cambio

Tabla I. Antígenos de *E. histolytica* clonados por reconocimiento con anticuerpos IgA de salivas.

Procedencia de la saliva usada en el tamizaje	**Clonas de cDNA aisladas**	**Identidad de las proteínas clonadas**
Pacientes con amibiasis intestinal	ZSP1	Proteína de 29 kDa rica en cisteínas (CREHP)
	ZSP6 y ZSP7	Proteína rica en serinas (SREHP)
	ZSP8	Factor 1-α de la elongación
Pacientes con absceso hepático amibiano	Saha1	Enolasa
	Saha2	Proteína de choque térmico hsp70 (HSP70)
	Saha3	Acetaldehído/alcohol deshidrogenasa (EhADH2)
	Saha4	Proteinasa de cisteínas
	Saha51	Proteína ribosomal L-23a (EhRP-L23a)
	Saha54	Ciclofilina EhCyp (EhCyp)
Portador asintomático	SalJC1	Proteína desconocida rica en ácido-glutámico EhSalJC1

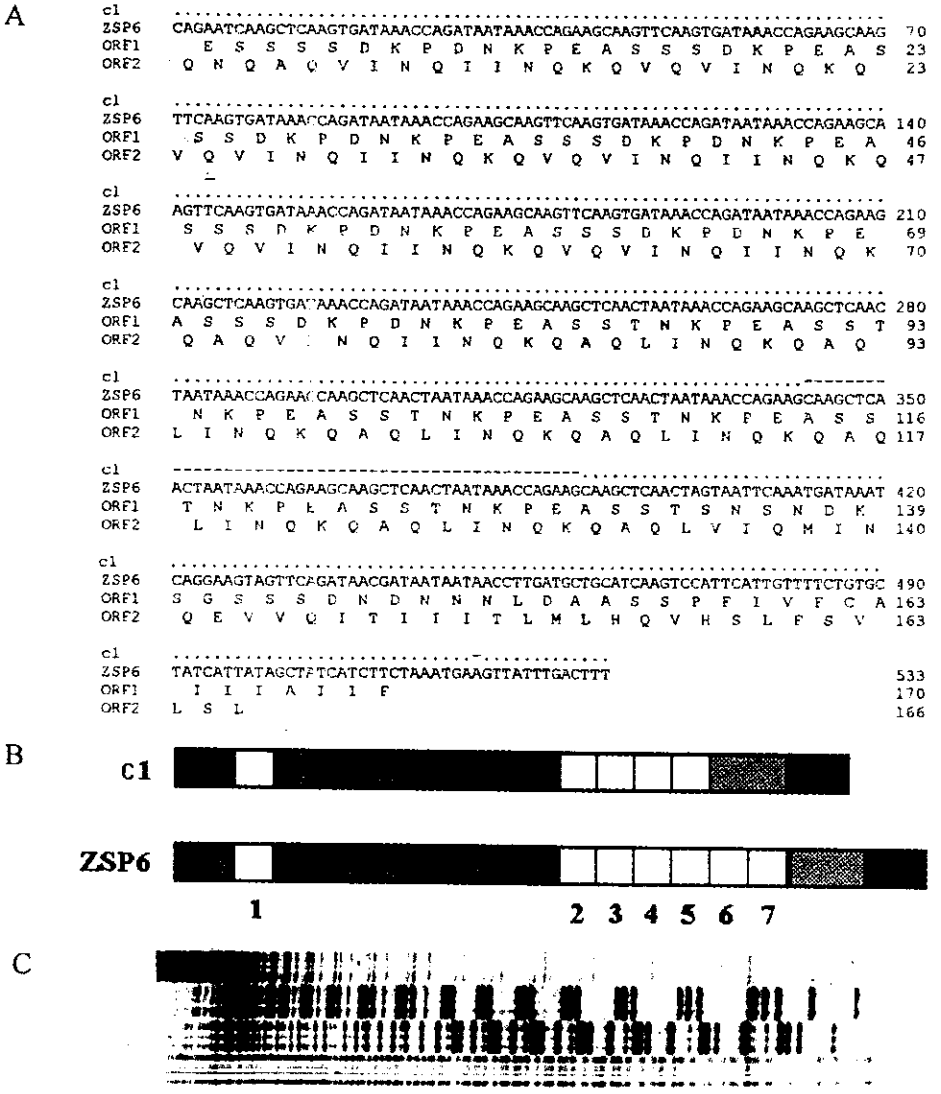


Fig. 7 Caracterización de la clona de cDNA Zsp6 que codifica para una nueva isoforma de la proteína rica en serinas de *E. histolytica* (SREHP). A) Secuencia de nucleótidos y las deducidas de aminoácidos para los dos ORFs que se sobrelapan. Comparación de la secuencia de nucleótidos de Zsp6 con aquella de la clona c1 que codifica para la SREHP reportada por el grupo de Stanley. Los puntos indican homologías; las líneas rotas indican los nucleótidos ausentes en c1. B) Representación esquemática de las SREHP derivadas de las clonas Zsp6 y c1, siguiendo el estilo de Stanley y col., 1990. Los números indican el número de repeticiones octapeptídicas. ■ Regiones hidrofóbicas; ▩ repeticiones de dodecapéptidos; □ repeticiones de octapéptidos. C) Segmento de las placa de secuencia mostrando el número de repeticiones de la SREHP clonada en este trabajo.

sencillo de un nucleótido en la posición 209 (A por G) que resulta en la substitución de un aminoácido (L por R) (Fig. 8). Finalmente, la cuarta clona caracterizada Zsp8, mostró un inserto de 291 bp con un ORF que codifica para un fragmento del factor 1-α de la elongación previamente reportado (Fig. 9A) (De Meester y col., 1991).

Clonas de cDNA aisladas con saliva de un paciente con AHA (clonas Saha). Se aislaron un total de 8 clonas de las cuales seis se caracterizaron. La clona Saha1 contenía un inserto de 327 bp que codifica para los 108 aminoácidos del extremo carboxilo-terminal de la enzima enolasa de la via glucolítica (Fig. 9B) (Beanan y Bailey., 1995). Otra clona aislada denominada Saha4, contenía un inserto de 941 bp incluyendo un ORF, que codifica una secuencia de 308 aminoácidos correspondiente a la proteinasa de cisteína de la amiba (Fig. 9C) (Reed y col., 1993). Los insertos de otras dos clonas (Saha2 y Saha3) codifican para péptidos con ligera homología a dos proteínas conocidas de la amiba. La clona Saha2 con un inserto de 708 bp codifica para un péptido de 232 aminoácidos el cual muestra entre 50-60% de similaridad a un fragmento interno de la proteína de choque térmico de 70 kDa (HSP70) descrita para la cepa SFL-3 de *E. histolytica* (Fig. 10A) (Ortner y col., 1992). A pesar de tratarse de proteínas sumamente conservadas, la diferencia de secuencias se podría deber a que son derivadas de diferentes cepas de *E. histolytica* (la utilizada en este estudio es HM1:IMSS). En cualquier caso, a nuestro conocimiento este es el primer indicio de una proteína tipo HSP70 en trofozoítos de la cepa HM1:IMSS. La clona Saha3 mostró un inserto de 592 bp que codifica para un péptido de 191 aminoácidos que presenta ligera homología (40-50%) con la enzima acetaldehido/alcohol deshidrogenasa (EhADH2; Fig. 10B) (Bruchhaus y Tannich., 1994). La identificación de una aparentemente nueva EhADH2, apoya la sugerencia de otros autores (Bruchhaus y Tannich., 1994) de la existencia en *E. histolytica* de una familia de proteínas análogas a los productos del gen multifuncional *adhE* de *E. coli* y distinta de las alcohol deshidrogenasas dependientes de NAD+ y NADP+ previamente reportadas.

Las secuencias del otro par de clonas, Saha51 y Saha54, codifican para dos proteínas no reportadas previamente en *E. histolytica*. La clona Saha51 contenía un inserto de 383 bp

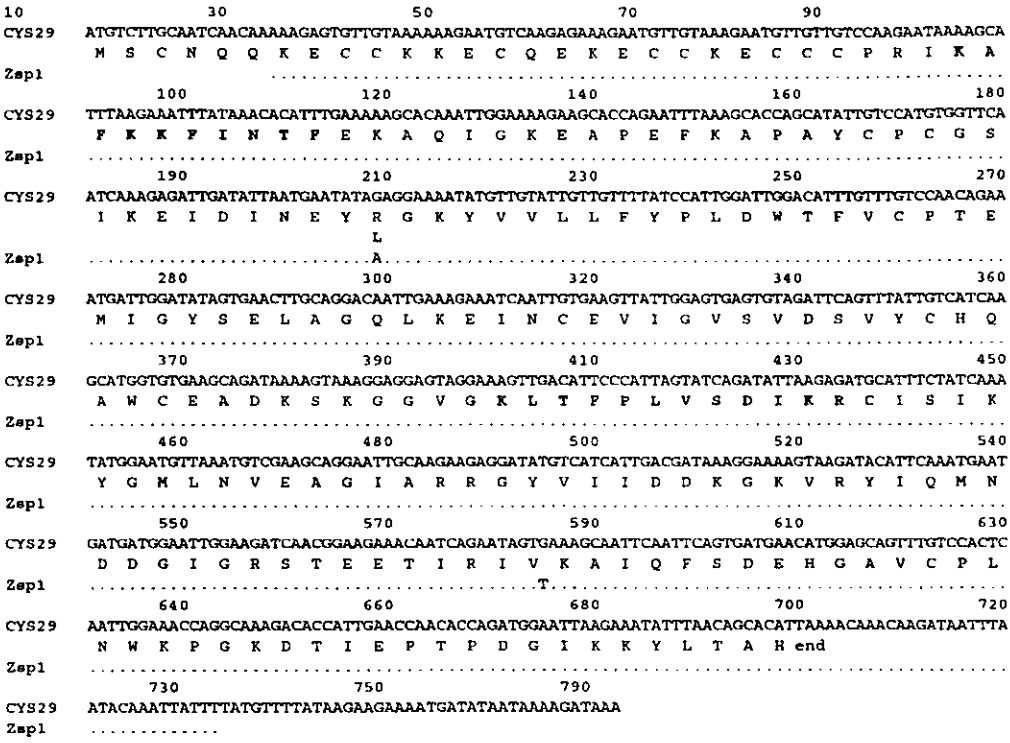


Fig. 8 Secuencia de nucleótidos y de aminoácidos de la clona de cDNA Zsp1. Análisis comparativo con la secuencia reportada para la proteína de 29 kDa rica en cisteínas de *E. histolytica*. Los puntos indican homologías; El residuo de leucina (L) en negrita indica el único cambio entre las secuencias; los dos segmentos de secuencia en negrita son predicciones de epítopes para células T (GeneWorks; IntelliGenetics).

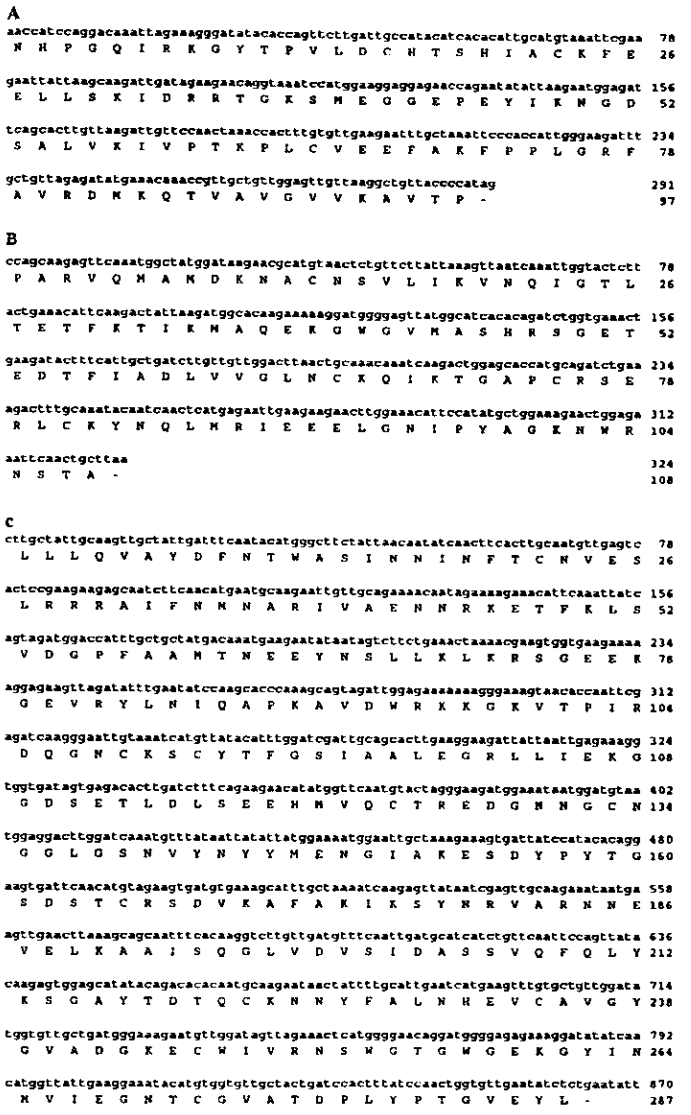


Fig. 9 Secuencia de nucleótidos y de aminoácidos de las clonas de cDNA caracterizadas con homología total a proteínas reportadas para *E. histolytica*. A) Clona Zsp8 que codifica para el factor 1-α de la elongación; B) Clona Saha1 que codifica la enolasa; C) Clona Saha4 que codifica la proteinasa de cisteína.

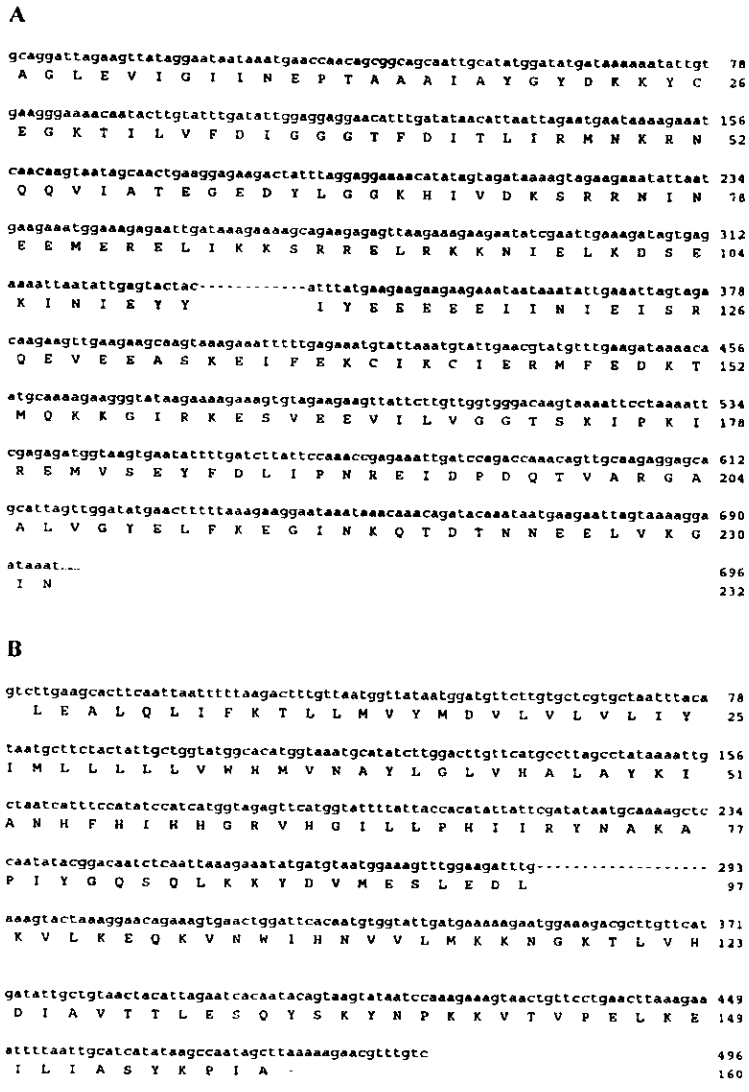


Fig. 10 Secuencia de nucleótidos y de aminoácidos de las clonas de cDNA caracterizadas que muestran homología parcial con proteínas reportadas para *E. histolytica*. A) Clona Saha2 que codifica para una proteína HSP70 con escasa homología a la reportada para la cepa SFL-3 de *E. histolytica*. B) Clona Saha3 que codifica para una acetaldehído/alcohol deshidrogenasa con escasa homología a las reportadas con anteriodad en la amiba (EhADH2).

con un ORF de 119 aminoácidos y una región no codificante 3´ de 21 bp (Fig. 11A). El análisis de Blast-p demostró que la secuencia deducida de aminoácidos corresponde a la proteína ribosomal L23a de *E. histolytica* (EhRP-L23a) mostrando 58% de identidad y 74% de similaridad a sus homólogos en humanos y rata. La proteína L23a es parte de un grupo de proteínas involucradas en la iniciación del ensamblaje de la subunidad ribosomal grande (El-Baradi y col., 1983). El fragmento de EhRP-L23a muestra la secuencia KKAFVRL en el extremo carboxi-terminal (posición 97-103) que recuerda al motivo altamente conservado (KKAYVRL) de las L23a involucrado en la unión al rRNA (Fig. 11B). El residuo de leucina terminal es crítico para la unión de la proteína L23a al dominio III del RNAr 25S (Rutgers y col., 1991). La otra proteína ribosomal descrita en *E. histolytica*, RP-L21, se encontró asociada a uno de los genes de actina en el genoma (Petter y col., 1992). Por su parte la clona Saha54 contenía un inserto pequeño de 242 bp, con un ORF codificante de 75 aminoácidos, seguido por una región corta no codificante de 15´ bp y del tallo de poli-A . La investigación de homologías en el servicio Blast-p de la secuencia de aminoácidos deducida, indicó que el inserto de Saha54 codifica para un péptido perteneciente a un grupo de proteínas altamente conservadas conocidas como Ciclofilinas (CyPs). Estas proteínas, junto con la proteína de unión a FK506 (FKBPs), constituyen la familia de inmunofilinas, enzimas con actividad de isomerasa cis-trans de peptidil-prolil las cuales actúan como receptores intracelulares de drogas inmunosupresoras como Ciclosporina A (CsA), FK506 y Rapamicina (Trandinh y col., 1992). La secuencia completa de EhCyp se obtuvo a partir de una clona genómica aislada con el inserto de la clona de cDNA. La clona genómica denominada Gsaha 54, contenía un inserto de 2 kb con el ORF completo de la EhCyp de 504 bp que codifican para una proteína de 168 aminoácidos con un peso molecular de 18,984 Da. El codón de terminación TAA se encuentra en la posición 501 con respecto al codón ATG marcado como la posición 1(Fig. 12A). El tamaño de EhCyp deducido de la secuencia de nucleótidos, correlaciona con el tamaño esperado de acuerdo a los resultados del Northern (ver adelante) y es similar al de otras Cyps reportadas. La secuencia de aminoácidos deducida, muestra todos los residuos conservados presentes en el extremo carboxilo-terminal de las CyPs, involucrados en la unión a la CsA. Este incluye un residuo de triptofano ubicado en la posición 141 que es esencial en dicha unión (Fig.

A.

```
CGGAACCAAGAGATATACAACTGCTTTCCATAGACCAGCTACAAAGAATGCTGCAAAAGCACCAAAATATCCAAGATCACTTAAAGCTAATAGAAATAAA  100
  G  T  K  R  Y  T  T  A  F  H  R  P  A  T  K  N  A  A  K  A  P  K  Y  P  R  S  L  K  A  N  R  N  K    33

ATGGACGAATTTTCCATTATTAAATTCCCACTCGCTACAGAAACTGCTCTTAAGAAAATCGAAGACCACAACACTCTTGTTTTCCTTTGTGAACAAAAAG  200
 M  D  E  F  S  I  I  K  F  P  L  A  T  E  T  A  L  K  K  I  E  D  H  N  T  L  V  F  L  C  E  Q  K     66

CTAATAAGACTATGATTAAGAAAGCTGTTGAAAAGAGATATGGAGTTAAAGTTATTAAAGTAAATACCCTTGTTAGACTTGATGGTCTTAAGAAAGCTTT  300
A  N  K  T  M  I  K  K  A  V  E  K  R  Y  G  V  K  V  I  K  V  N  T  L  V  R  L  D  G  L  K  K  A  F  100

CGTTAGACTTGCACCAGATGTAGAAGCTATGGAAGTTGCAACCAAGATTGGACTCTTCTAAGAAAATGGTTCTATTGTGGTTAAAAAAAAAAAAAAAAAA  400
  V  R  L  A  P  D  V  E  A  M  E  V  A  T  K  I  G  L  F  *                                          119
```

B.

```
EhRP-L23a  G T K R Y T T A F H R P A T K N A A K A P K Y P R S L K A N
Human      K K I R T S P T F R R P K T L R L R R Q P K Y P R K S A P R
Rat        K K I R T S P T F R R P K T L R L R R Q P K Y P R K S A P R
Tobacco    K K I R T K V T F H R P K T L K K D R N P K Y P R I S A P G

EhRP-L23a  R N K M D E F S I I K F P L A T E T A L K K I E D H N T L V
Human      R N K L D H Y A I I K F P L T T E S A M K K I E D N N T L V
Rat        R N K L D H Y A I I K F P L T T E S A M K K I E D N N T L V
Tobacco    R N K L D Q Y G I L K Y P L T T E S A M K K I E D N N T L V

EhRP-L23a  F L C E Q K A N K T M I K K A V E K R Y G V K V I K V N T L
Human      F I V D V K A N K H Q I K Q A V K K L Y D I D V A K V N T L
Rat        F I V D V K A N K H Q I K Q A V K K L Y D I D V A K V N T L
Tobacco    F I V D I K A D K K K I K D A V K K M Y D I Q T K K V N T L

EhRP-L23a  V R L D G L K K A F V R L A P D V E A M E V A T K I G L F *
Human      I R P D G E K K A Y V R L A P D Y D A L D V A N K I G I I *
Rat        I R P D G E K K A Y V R L A P D Y D A L D V A N K I G I I *
Tobacco    I R P D G T K K A Y V R L T P D Y D A L D V A N K I G I I *
```

Fig. 11 Caracterización de la clona de cDNA Saha51 que codifica para la proteína ribosomal L-23a (EhRP-L23a), una proteína no descrita con anterioridad en *E. histolytica*. A) Secuencia de nucleótidos y de aminoácidos; B) Análisis de homología con las secuencias de otros organismos eucariotes. El motivo conservado que permite la unión de las RP-L23a al rRNA está enmarcado en un recuadro doble. El residuo leucina esencial en la unión está señalado con una flecha.

A

```
ctctcactgatgtgatgagaaataagctagtatcagatgatactgctgtgtatcaagaatctcatggatggtaaagtg -230
atatactatatcatacaatcagtatgtattcatatatttatgttcttttattgttaaaattgttatttttttataact -152
tagtaattgataaaattatatttaagatctttttttgttttatctttttaagatttttatattctaaaaatgacaaa  -74
aatgaaacacgtcatgccacggagaggagatgtattaaaggatctttgaagaaatgataaaaattattgataaa       -1
atggcaagaccaaaggtattctttgatattactattggaggtgaaaaagctggtagaattgttatggaactctttaac   78
 M  A  R  P  K  V  F  F  D  I  T  I  G  G  E  K  A  G  R  I  V  M  E  L  F  N    26

gatattgtcccaaagacagctgaaaacttcagatgcctttgcactggtgaaaagggtaatggacttacctataagggt  156
 D  I  V  P  K  T  A  E  N  F  R  C  L  C  T  G  E  K  G  N  G  L  T  Y  K  G    52

tgtggattccatagagttattaaggatttcatgattcaaggaggtgatttcactagacataatggaactggaggaaaa  234
 C  A  F  H  R  V  I  K  D  F  M  I  Q  G  G  D  F  T  R  H  N  G  T  G  G  K    78

tcaatttatggtactaagtttgctgatgaagctttcactgttaaacacactaaaccaggaatgttatctatggctaat  312
 S  I  Y  G  T  K  F  A  D  E  A  F  T  V  K  H  T  K  P  G  M  L  S  M  A  N   104

gctggaccaaatactaatggttcacaattctttattaccactgttccatgcccatggttagatggtaaacatgttgtc  390
 A  G  P  N  T  N  G  S  Q  F  F  I  T  T  V  P  C  P  W  L  D  G  K  H  V  V   130

tttggtcaagttgttgaaggttatgatgttgttaaaatgatcgaaaacaacccaactggtgctcaagacaaaccaaag  468
 F  G  Q  V  V  E  G  Y  D  V  V  K  M  I  E  N  N  P  T  G  A  Q  D  K  P  K   156

aaagccgttgttattgctgattgtggtcaattataaatgattacaattttgtctc                        523
 K  A  V  V  I  A  D  C  G  Q  L  -                                            167
```

B

```
                                                                              70
EhCyp       ---------- --MARPKVFF DITIGGEKAG RIVMELFNDI VPKTAENFRC LCTGEKGNG. .....LTYKG
CYPH_MAIZ   ---------- --MANPRVFF DMTVGGAPAG RIVMELYANE VPKTAENFRA LCTGEKGVG. KSGKPLHYKG
CYPH_LYCES  ---------- --MANPKVFF DLTIGGAPAG RVVMELFADT TPKTAENFRA LCTGEKGVG. KMGKPLHYKG
CYPH_BLAGE  ---------- --MAHPRVFF DMSADGQPVG RIVMELRSDV VPKTAENFRA LCTGEKGFG. .......YKG
CYP4_BOVIN  ---------- -------VFF DVDIGGEXVG RXXLELFADI VPKTAENFRA LCTGEKGIGP TTGKPLHFKG
CYP4_HUMAN  MSHPSPQAKP SNPSNPRVFF DVDIGGERVG RIVLELFADI VPKTAENFRA LCTGEKGIGH TTGKPLHFKG
CYP1_ARATH  ---------- --MAFPKVYF DMTIDGQPAG RIVMELYTDK TPRTAENFRA LCTGEKGVG. GTGKPLHFKG
 Consensus  ********** **------*-* *----*---* *---**---- -*-*******- ********-*- ---------**

                                                                             140
EhCyp       CAFHRVIKDF MIQGGDFTRH NGTGGKSIYG TKFADEAFTV KHTKPGMLSM ANAGPNTNGS QFFITTVPCP
CYPH_MAIZE  STFHRVIPEF MCQGGDFTRG NGTGGESIYG EKFPDEKFVR KQPAPGVLSM ANAGPNTNGS QFFICTVATP
CYPH_LYCES  STFHRVIPGF MCQGGDFTAG NGTGGESIYG AKFNDENFVK KHTGPGILSM ANAGPGTNGS QFFICTAKTE
CYPH_BLAGE  SRFHRVIPNF MCQGGDFTNH NGTGGKSIYG TKFEDENFQL KHTGPGILWM ANAGPNTNGS QFFITTAKTS
CYP4_BOVIN  CPFHRIIKKF MIQGGDFSNQ NGTGGESIYG EKFEDENFHY KHDKEGLLSM ANAGSNTNGS QFFITTVPTP
CYP4_HUMAN  CPFHRIIKKF MIQGGDFSNQ NGTGGESIYG EKFEDENFHY KHDREGLLSM ANAGRNTNGS QFFITTVPTP
CYP1_ARATH  SKFHRVIPNF MCQGGDFTAG NGTGGESIYG SKFEDENFER KHTGPGILSM ANAGANTNGS QFFICTVKTD
 Consensus  --****-*--* *-*****--- *****-**** -**-**-*-- *-----*-*- ******-**** ****-*----

                                                                             190
EhCyp       WLDGKHVVFG QVVEGYDVVK MIENNPTGAQ DKPKKAVVIA DCGQL
CYPH_MAIZE  WLDGKHVVFG QVVEGMDVVK AIEKVGT.RN GSTSKVVKVA DCGQLS
CYPH_LYCES  WLNGKHVVFG QVVEGMDVIK KAEAVGS.SS GRCSKPVVIA DCGQL
CYPH_BLAGE  WLDNRHVVFG SVVEGMDVVK KLESLGS.QS GKTNKKIAVV DCGQI
CYP4_BOVIN  HLDGKHVVFG QVIKGMGVAK ILENV.EVKG EKPAKLCVIA ECGELKEGDD
CYP4_HUMAN  HLDGKHVVFG QVIKGIGVAR ILENV.EVKG EKPAKLCVIA ECGELKEGDD
CYP1_ARATH  WLDGKHVVFG QVVEGLDVVK AIEKVGS.SS GKPTKPVVVA DCGQLS
 Consensus  -*----***** -*---*--*-- --*------- ----*----- -**--*****
```

Fig. 12 Caracterización de la clona genómica Gsaba54 con un ORF que codifica para la primera ciclofilina descrita en *E. histolytica* (EhCyp). A) Secuencia completa de nucleótidos y de aminoácidos que codifica para EhCyp (número de acceso AF017993); los codones de inicio (ATG) y del final (TAA) de la traducción están indicados en negrita. Subrayados se muestran tres elementos en la región promotora del gen involucrados en el control de la transcripción (Ref. 75). B) Análisis de homología con las secuencias de otros organismos. Los residuos involucrados en la unión de las ciclofilinas a la droga CsA están indicados con puntos. El residuo triptofano esencial en la unión está señalado con una flecha. Lyces: *Lycopersicon esculentum* (tomate); Blage: *Blatella germánica* (insecto); Arath: *Arabidopsis thaliana* (planta).

12B; Liu y col., 1991; Trandinh y col., 1992). La EhCyp como la mayoría de las ciclofilinas es una proteína altamente conservada como lo demostró su alta homología con la mayor parte de las Cyps reportadas, incluyendo la humana y la bovina (Fig. 12B).

Clonas de cDNA aisladas con la saliva de un portador asintomático (clonas SalJC). Con la saliva del portador asintomático, se aislaron dos clonas idénticas (SalJC1 y 2) de la biblioteca de cDNA. Los insertos de las clonas de 525 bp mostraron un ORF que codifica para un péptido de 175 aminoácidos que carece de codón de detención (EHSalJC1 protein; Fig. 13). Un segundo tamizaje de la biblioteca de cDNA utilizando el inserto de SalJC1como sonda para aislar clonas completas, resultó en el aislamiento de 10 clonas con insertos aún más pequeños que el de SalJC1 y que analizados por secuenciación corresponden efectivamente al mismo gen. El análisis de la secuencia de una onceava clona aislada mostró que codifica para otra proteína desconocida, cuya única similitud con la secuencia de SalJC1 es la presencia de una serie de motivos nucleotídicos ricos en G y A (GAG; GAAG y GAAAG). El análisis de homología basado en la secuencia de aminoácidos deducida de SalJC1 demostró que ésta no muestra similitud significativa con ninguna proteína reportada para *E. histolytica,* ni para ningún otro organismo. Así, la proteína codificada por SalJC1 (EHSalJC1) puede corresponder a una nueva proteína o parte de una nueva familia de proteínas aun no caracterizadas. La secuencia de nucleótidos de SalJC1, al igual que la mayoría de las secuencias reportadas en *E. histolytica*, muestra un alto contenido de A/T (66%). Por su parte, EHSalJC1 muestra un contenido elevado de residuos de ácido glutámico (14.3%) y el análisis computacional, usando el programa GeneWorks (IntelliGenetics), reveló un sitio potencial de N-glicosilación en la posición 72 y predijo dos fragmentos de secuencia similares a epítopes de células T (Fig. 13). Actualmente se están haciendo esfuerzos para aislar clonas completas de esta o estas proteínas a partir de una biblioteca genómica de *E. histolytica.*

```
        10                  30                  50                  70
ATTTTTGGGAATAAACCAATCATGGGAGGACCAGCACCAATAAGACCAAAGCCAGTGGAACAGAAAAATGAAGAAAGTAA
 I  F  G  N  K  P  I  M  G  G  P  A  P  I  R  P  K  P  V  E  Q  K  N  E  E  S  N

        90                  110                 130                 150
TGAAGAGACAATCGAGATTCGACATGATGGTAAAATTAGTAGAAGAAGTGTATCAGGTCCAAAAGGAAGAAGAAGACCAA
  E  E  T  I  E  I  R  H  D  G  K  I  S  R  R  S  V  S  G  P  K  G  R  R  R  P

        170                 190                 210     *           230
CAATGAAAAAGATTCAATTTGATGAACATACTGAACGACTCATGAAAGAAATCAATGACAATAAGACACAGGAAAGCAAC
T  M  K  K  I  Q  F  D  E  H  T  E  R  L  M  K  E  I  N  D  N  K  T  Q  E  S  N

        250                 270                 290                 310
AAAGAAAGGATTCAAGATTTGTTAGATGGCAAAGAAGTAAAACCTATCAGAACAGATGACATTAAACACCAAGAGGTTGA
 K  E  R  I  Q  D  L  L  D  G  K  E  V  K  P  I  R  T  D  D  I  K  H  Q  E  V  D

        330                 350                 370                 390
TGAATCAACAAAAGAAGTTCATGTATTAGACAAAGAAGAAAAGGAAGATTCAGGGGATAGTGATGGTTTTGTTATTGAAG
  E  S  T  K  E  V  H  V  L  D  K  E  E  K  E  D  S  G  D  S  D  G  F  V  I  E

        410                 430                 450                 470
ATGATCAACCATCAAGCACTACAAATATTAGTGCCAAAGAGAATGATATTAAAGAAAATGATACTGAACATAAGGAACAA
D  D  Q  P  S  S  T  T  N  I  S  A  K  E  N  D  I  K  E  N  D  T  E  H  K  E  Q

        490                 510
GTAAAGAGTGATGACACAACAGTAATAGATGAAATATTTGAATCGAAAAAAAAAAAAAAA
 V  K  S  D  D  T  T  V  I  D  E  I  F  E  S  K  K  K  K
```

Fig. 13 Secuencia de nucleótidos y de aminoácidos de la clona de cDNA SalJC1 (número de acceso U66671). EHSalJC1 muestra un contenido elevado de residuos de ácido glutámico (14.3%). Un sitio potencial de N-glicosilación se indica con un asterisco; los dos segmentos de secuencia en negrita son predicciones de epítopes para células T (GeneWorks; IntelliGenetics).

Tamaño de los transcritos de EhCyp, EHSalJC1 y EhRP-L23a. Con la finalidad de determinar el tamaño real de las tres proteínas no reportadas previamente en *E. histolytica*, se realizó un análisis de Northern blot sobre el RNAm de trofozoítos, utilizando como sondas los insertos de las clonas Saha54, Saha51 y SalJC1. Para EhCyp y EhRP-L23a se detectaron transcritos sencillos de 0.6 y 0.45 kb respectivamente, indicando con base a la secuencia total determinada en clonas aisladas de una biblioteca genómica, que los genes de estas proteínas carecen de intrones, como se ha observado en la mayoría de las secuencias codificadoras reportadas para *E. histolytica* (sólo se han reportado intrones muy cortos en dos genes de la amiba) y que estas proteínas parecen derivarse de un mensajero único (Fig.14; carriles A y B). Por el contrario, el inserto de la clona SalJC1 hibridó por lo menos con 4 transcritos de RNAm con tamaños que van desde 0.45 a 1.5 kb (0.45-0.6, 0.8, 1.2 y 1.5 kb aproximadamente), sugiriendo que EHSalJC1 podría formar parte de una familia amplia de proteínas relacionadas (Fig. 14; carril C). Los tamaños identificados para los transcritos de EhCyp y EhRP-L23a son consistentes con aquéllos descritos para sus homólogos en otros parásitos (Reddy, 1995; Suzuki y Wool., 1993).

Expresión recombinante y purificación de EHSalJC1 y de la proteína de 29 kDa rica en cisteínas de E. histolytica (CREHP). Los insertos completos de las clonas Saljc1 y Zsp1 que codifican para el péptido EHSalJC1 y la CREHP respectivamente, se subclonaron en marco dentro del vector de expresión pRSET. Después de la transformación, las células XL1-Blue se indujeron a expresar los péptidos de *E. histolytica* como productos de fusión, con un tallo de poli-histidinas que se une a metales. Un análisis cinético de expresión de las proteínas recombinantes por corrimiento de un lisado de bacterias inducidas y recolectadas cada hora a partir de la adición de IPTG, indicó que las proteínas recombinantes se comenzaron a expresar a las 2h post-inducción y que la maxima expresión se observó a las 5 horas (Fig. 15A). Las proteínas de fusión purificadas mostraron una pureza de más del 90% (Fig. 15B), así como los tamaños esperados de 24 kDa (EhSalJC1) y 29 kDa (CREHP) determinados por SDS-PAGE (Fig. 16A).

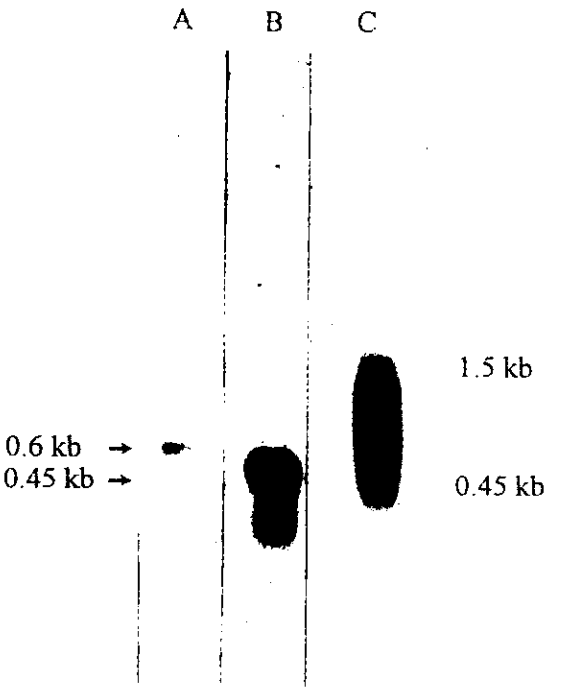


Fig. 14 Análisis tipo Northern blot de las clonas de cDNA aisladas que codifican para proteínas no reportadas previamente en *E. histolytica*. RNAm de trofozoítos hibridado con los insertos completos de las clonas Saha54 (carril A), Saha51 (carril B) y SalJC1 (carril C) que codifican para EhCyp, EhRP-L23a y EHSalJC1, respectivamente.

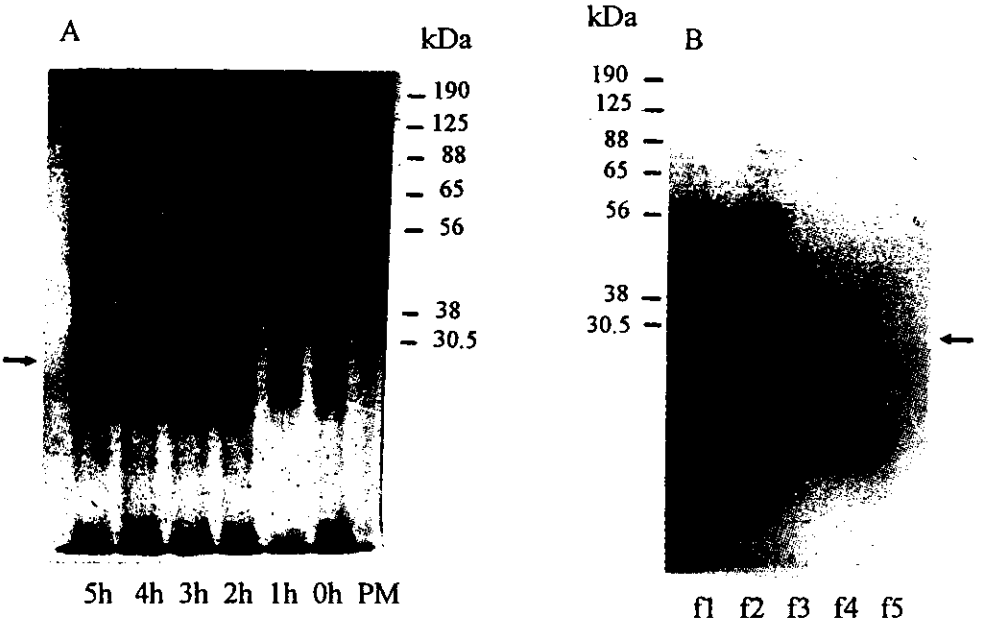


Fig. 15 Cinética de expresión y purificación de la proteína recombinante de 29 kDa CREHP. A) Muestras de bacterias transformadas con la construcción pRSET$_B$-Zsp1 se recolectaron cada hora después de la adición del IPTG y del fago M13/T7. Un lisado de las muestras se sometió a electroforesis en SDS-PAGE al 12 % (20 µg/carril) y se tiño con azul de coomasie. La CREHP inducida está señalada con una flecha. PM: marcador de pesos moleculares. B) Electroforesis en SDS/PAGE al 12 % de las fracciones eluídas (5 µg/carril) de una columna tratada con niquel y cargada con la fracción soluble de bacterias transformadas con pRSET$_B$-Zsp1 e inducidas con IPTG durante 5 h. La proteína recombinante de CREHP señalada con la flecha, se eluye con el primer ml del eluyente (f1 y f2).

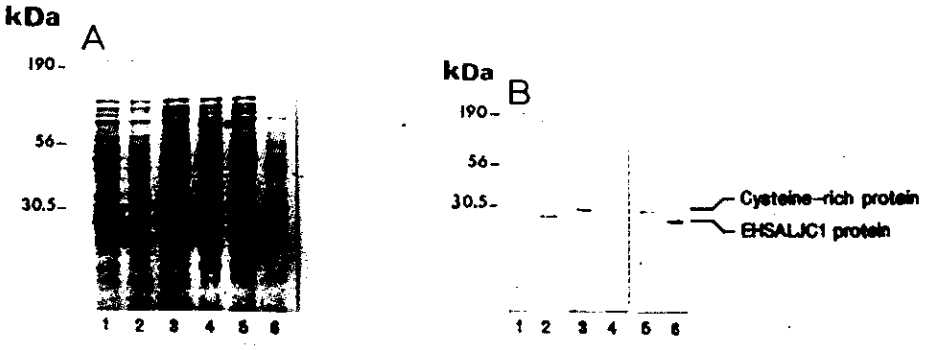


Fig. 16 Análisis de expresión y antigenicidad de los péptidos recombinantes CREHP y EHSaIJC1. A) Electroforesis en SDS-PAGE al 12 % de lisado de bacterias XL1-Blue transformadas con las construcciones e inducidas (carriles 1 y 4), transformadas pero no inducidas (carriles 2 y 5) y transformadas con el plásmido sin inserto e inducidas (carriles 3 y 6). EHSaIJC1: carriles 1-3, CREHP: carriles 4-6. Las péptidos inducidos están señaladas por cabezas de flechas. Una banda de naturaleza desconocida está indicada por un punto. B) Inmunotransferencia de los péptidos purificados recombinantes CREHP (carriles 1,3 y 5) y EHSaIJC1 (carriles 2,4 y 6) con la IgA de saliva (dil 1:2) de pacientes con amibiasis intestinal (carriles 1 y 2), de un portador asintomático (carriles 3 y 4) y de un paciente con absceso hepático amibiano (carriles 5 y 6).

Reconocimiento de los productos recombinantes por anticuerpos IgA. Se realizaron ensayos de inmunotransferencia con las muestras de saliva usadas durante el tamizaje de la biblioteca, con la finalidad de determinar si los péptidos recombinantes purificados de CREHP y EHSaIJC1 preservaban los epítopes que reconocen los anticuerpos IgAs que permitieron su aislamiento. La CREHP, aislada con una mezcla de salivas de pacientes con amibiasis intestinal, fue reconocida por este tipo de muestra pero no por la saliva del portador asintomático (Fig. 16B). De igual forma, la EHSaIJC1 aislada con la saliva del portador asintomático, fue reconocida sólo por esta saliva. Sin embargo, ambas proteínas fueron reconocidas por IgA de la saliva del paciente con AHA. Estos resultados puede reflejar diferencias en el reconocimiento de antígenos por el sistema inmune de mucosas de pacientes y de sujetos portadores sanos.

Estudios de protección. Se realizaron ensayos de protección con las proteínas CREHP y EHSaIJC1 en un modelo murino de amibiasis intestinal que se asemeja a su contraparte en humanos (Ghosh y col., 1994) como una evaluación inicial del potencial protector de una inmunización oral con los fragmentos recombinantes de proteínas amibianas identificadas con anticuerpos IgAs. La administración por vía oral de cada uno de los fragmentos recombinantes purificados, acompañados con TC, indujeron intensas respuestas secretoras y séricas contra extracto total de amiba en los ratones C3H/HeJ al término del protocolo de inmunización (día 28, antes del reto con los trofozoítos). La respuesta sérica permaneció alta hasta el día del sacrificio (día 48), mientras que la respuesta secretora disminuyó considerablemente. El grupo control que sólo recibió TC no desarrolló ninguna respuesta contra el lisado de *E. histolytica* (Fig.17). Resultados similares se observaron en los sobrenadantes de cultivos de placas de Peyer. Análisis de inmunotransferencia sobre lisado total y fracción de membranas de trofozoítos mostró que los sueros de los ratones inmunizados con la proteína recombinante CREHP reconocen una proteína de 29 kDa mientras que los sueros de los inmunizados con EHSaIJC1 reconocen cuatro proteínas de 145, 128, 54 y 30 kDa (Fig. 18).

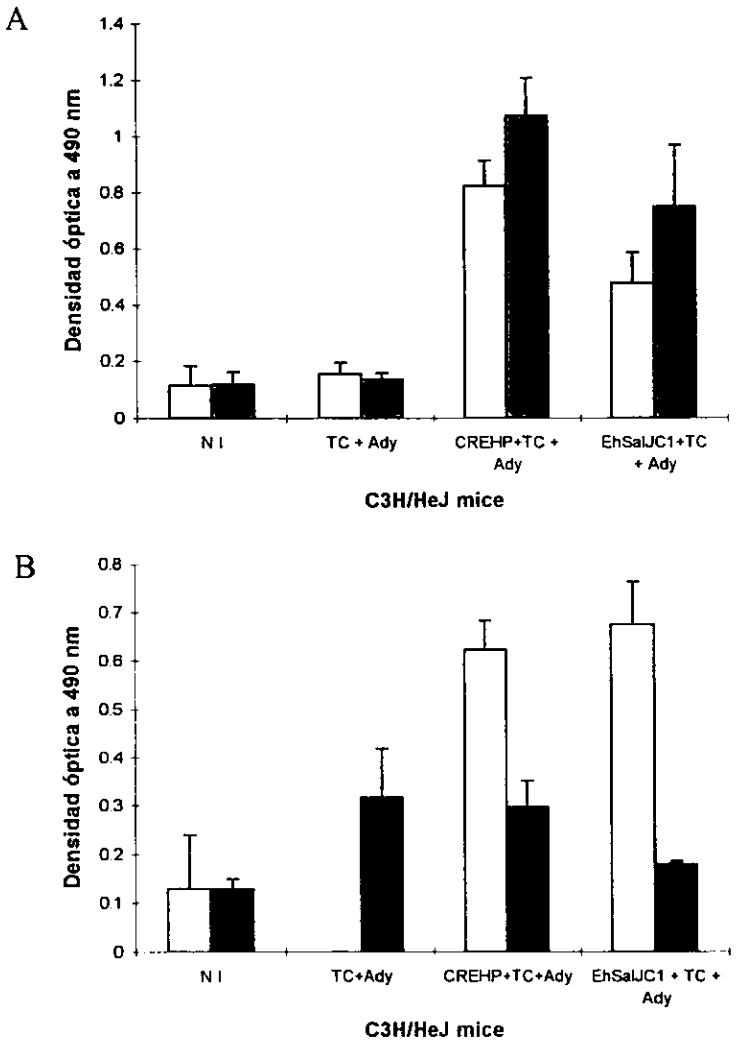


Fig. 17 Respuestas secretora y sistémica contra lisado de trofozoítos de *E. histolytica* de ratones C3H/HeJ inmunizados por vía oral con las péptidos recombinantes CREHP y EHsalJC1. A) Respuesta de anticuerpos anti-amiba determinada por ELISA en los sueros de los ratones al día 25 (barras blancas; antes de la infección) y día 48 (barras grises; día del sacrificio) después de la inmunización inicial. B) Respuesta de anticuerpos IgA anti-amiba en sobrenadantes de las heces de ratones al día 25 (barras blancas; antes de la infección) y 48 día (barras negras; día del sacrificio) despúes de la inmunización inicial. Las barras representan la media aritmética ± la desviación estándar del título de anticuerpos de 5 ratones. NI: no inmunizados; TC: tóxina del cólera; Ady: adyuvante incompleto de Freund.

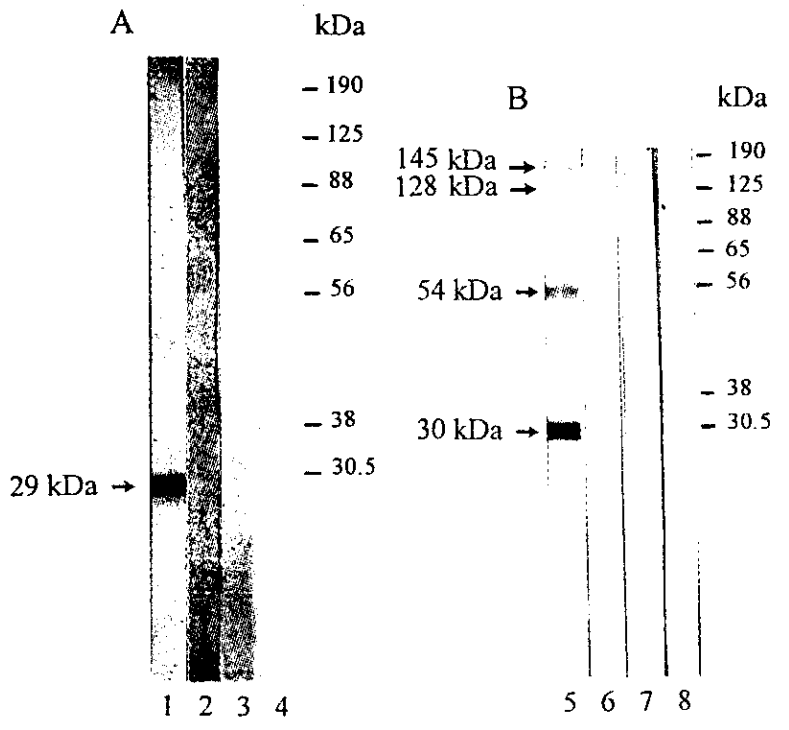


Fig. 18 Inmunotransferencia de lisado total de trofozoítos de *E. histolytica* con los sueros de los ratones C3H/HeJ inmunizados oralmente. A) Inmunotransferencia con la mezcla de sueros de los ratones inmunizados con la proteína recombinantes CREHP (carril 1); mezcla de sueros de ratones infectados pero no inmunizados (Carril 2); suero de ratón no inmune (carril 3) y control de reactivos (carril 4). B) Inmunotransferencia con la mezcla de sueros de los ratones inmunizados con el péptido EHSalJC1 (carril 5); mezcla de sueros de ratones infectados pero no inmunizados (carril 6); suero de ratón no inmune (carril 7) y control de reactivos carril 8).

A través del análisis coproparasitoscópico se demostró la presencia de trofozoítos en las heces de la mayoría de los ratones del grupo III (tratados con los adyuvantes e infectados), aún 20 días después de la infección. Por otra parte, la presencia de trofozoítos fue sólo ocasional en las heces de los ratones de los grupos inmunizados (grupo I:EhSalJC1 y grupo II: CREHP). La observación macroscópica de los ciegos y del contenido cecal demostró claras diferencias: los ciegos de los ratones del grupo III se observaron edematosos, con abundante irrigación y de color claro; el contenido cecal era mucoide, amarillento y fétido. Por el contrario, los ciegos de los ratones de ambos grupos inmunizados se mostraron similares a los del grupo de ratones sanos en tamaño y apariencia. Igualmente el contenido cecal era pastoso, oscuro y de olor *sui generis* (Fig.19A). El análisis microscópico del contenido cecal mostró la presencia de trofozoítos muy activos en los ratones del grupo control (III) y la ausencia de los mismos de los grupos inmunizados (I y II) (Fig. 19B y C).

El análisis histopatológico de las preparaciones de los ciegos de los ratones también demostró claras diferencias entre los los inmunes y los no inmunizados. El epitelio intestinal de los ratones infectados no inmunes mostró grandes infiltrados de neutrófilos y linfocitos, principalmente en la submucosa y en la lámina propia, con necrosis de la mucosa y destrucción completa del epitelio intestinal (Fig. 20A). De igual forma se detectó la presencia de trofozoítos entre los infiltrados celulares, de acuerdo con reportes previos demostrando la capacidad de la amiba para soslayar las barreras físicas del intestino (Fig. 20B). A pesar de que se observaron en los ciegos de los ratones del grupo II (inmunizados con EHSalJC1)algunos infiltrados ligeros, no se observó, al igual que los del grupo I, la presencia de trofozoítos. Así, mientras los ciegos de los ratones inmunizados con EHSalJC1 mostraron infiltrados escasos y pequeños, con poca evidencia de daño superficial del epitelio intestinal (Fig. 20D), los cortes de los ciegos de los ratones inmunizados con CREHP (Fig. 20C) fueron indistinguibles de aquellos de los ratones del grupo sano (IV).

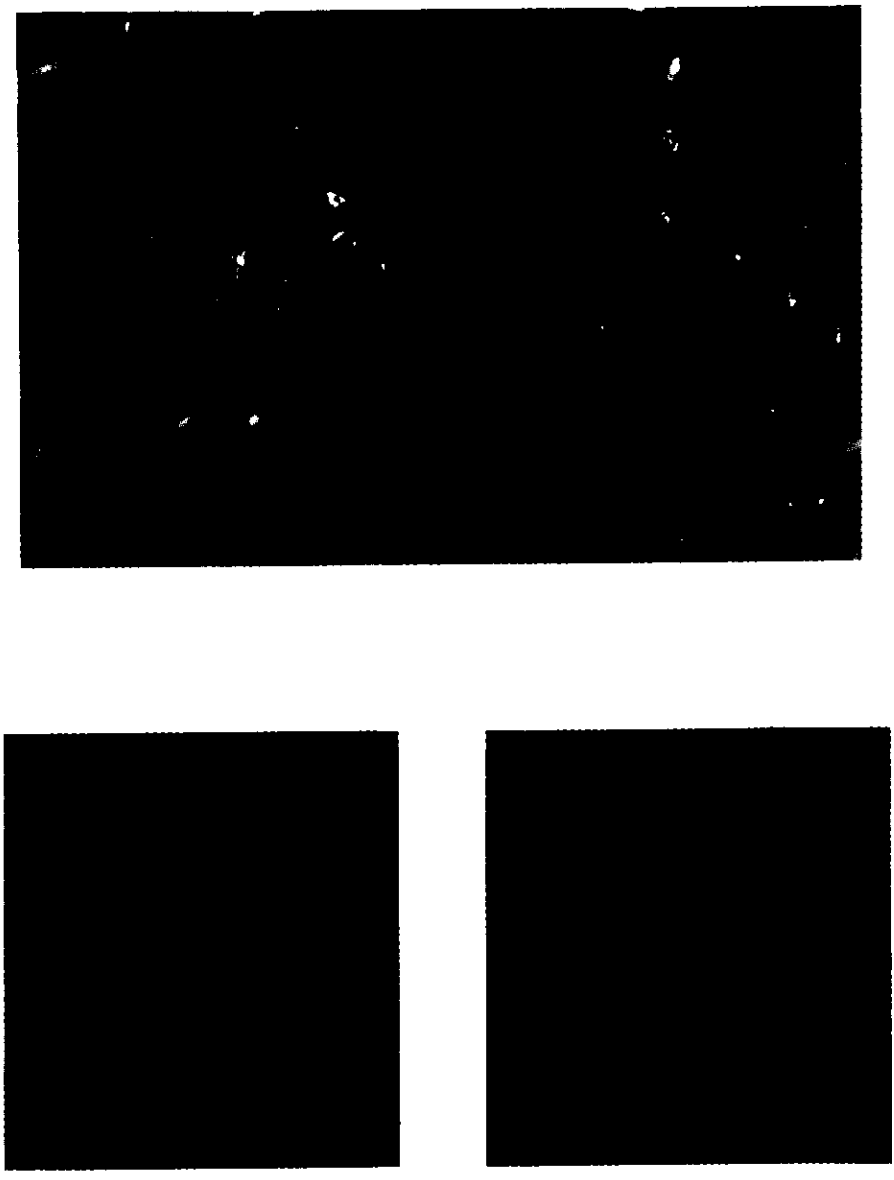


Fig. 19 Análisis macroscópico de los ciegos y microscópico del contenido cecal de los ratones C3H/HeJ infectados. A) Apariencia externa de los ciegos de un ratón no inmunizado (NI) y de un ratón inmunizado con la proteína recombinante CREHP (I). B y C) Tomas fotográficas de trofozoítos presentes en el contenido cecal y en las heces de un ratón no inmunizado al día del sacrificio, respectivamente (40X).

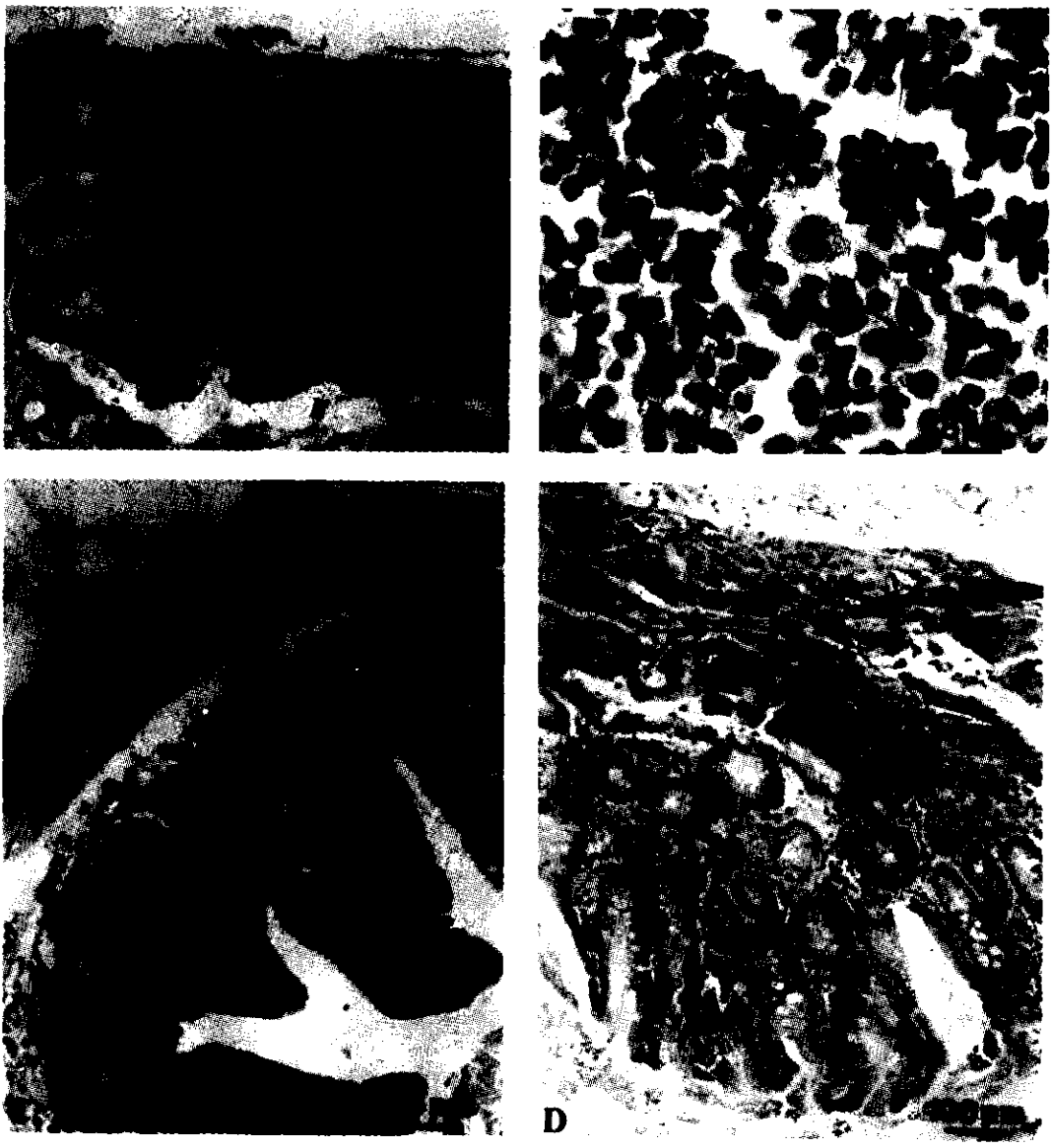


Fig. 20 Análisis microscópico de secciones de tejido cecal de ratones C3H/HeJ inmunizados y no inmunizados por vía oral y retados con trofozoítos de *E. histolytica*. A) Infiltrado extensivo de neutrófilos y linfocitos debajo del epitelio intestinal (flecha) en el ciego de un ratón no inmunizado (grupo III); e: epitelium. B) Amplificación del infiltrado inflamatorio anterior mostrando un trofozoíto activo. C) Ciego de un ratón inmunizado con la proteína recombinante CREHP. D) Ciego de un ratón inmunizado con el péptido recombinante EHSalJC1. Todas las preparaciones se tiñeron con hematoxilina-eosina.

Detección de actividad de PPIasa inhibible por CsA en extractos de E. histolytica. Se determinó la actividad de rotamasa en una fracción citosólica de *E. histolytica.* En este ensayo se usa quimotripsina para romper un péptido cromógeno, que sólo puede ser roto cuando se encuentra en la conformación *trans* (Fisher y col., 1984). El 80% del cromógeno en esa conformación es roto en los primeros segundos de la reacción, para luego disminuir bruscamente debido a una disminución del cambio de configuración y por consiguiente de la ruptura. La adición de cantidades crecientes del extracto citosólico conllevó a incrementos detectables en el nivel de ruptura del cromógeno (Fig. 21). Esta actividad se inhibió casi en su totalidad agregando a la mezcla de reacción concentraciones nanomolares de CsA. Ya que la CsA inhibe exclusivamente la actividad PPIasa debida a la ciclofilina, la inhibición de casi el 100 % sugiere que la mayor parte de la actividad es debida a una ciclofilina presente en trofozoítos de *E. histolytica.*

Efecto de la CsA sobre la proliferación de E. histolytica. La CsA mostró un marcado efecto inhibitorio sobre la proliferación y viabilidad de los trofozoítos dependiente de la concentración de la droga. A concentraciones bajas el efecto es primariamente inhibitorio, que eventualmente lleva a la muerte de los trofozoítos a las 48-72 h de cultivo. Sin embargo, a concentraciones elevadas el efecto es letal desde el inicio. El 50% de inhibición de la proliferación (IC_{50}) se observó en cultivos tratados con 1 a 10 μg/ml de CsA durante 48 h. La inhibición total de los trofozoítos se observó en los cultivos tratados desde el inicio con 50 μg/ml de CsA (Fig. 22). Las concentraciones de CsA entre 10^{-3} y 10^{-1} μg/ml mostraron un efecto débil pero lineal sobre la replicación de los trofozoítos. Bajo microscopía de luz, los trofozoítos tratados se observaron redondeados, sin movilidad y sin capacidad de adherencia a los tubos de cultivo, pero con la membrana plasmática aparentemente intacta. La integridad de la membrana plasmática así como de otros elementos internos en los trofozoítos tratados con CsA (ej. el núcleo), se confirmó por microscopía electrónica. Sólo se observó la presencia de una gran cantidad de gránulos densos distribuidos por todo el citoplasma (Fig. 23).

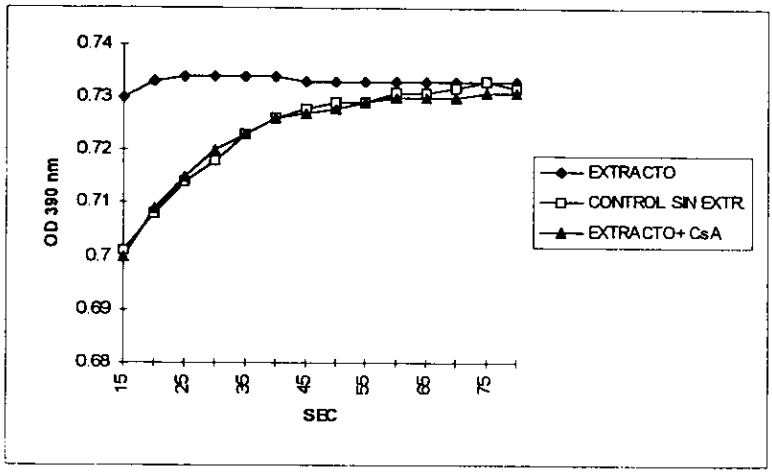


Fig. 21 Detección de actividad PPIasa en un extracto citosólico de trofozoítos de *E. histolytica*. Control se refiere al ensayo en ausencia de extracto.

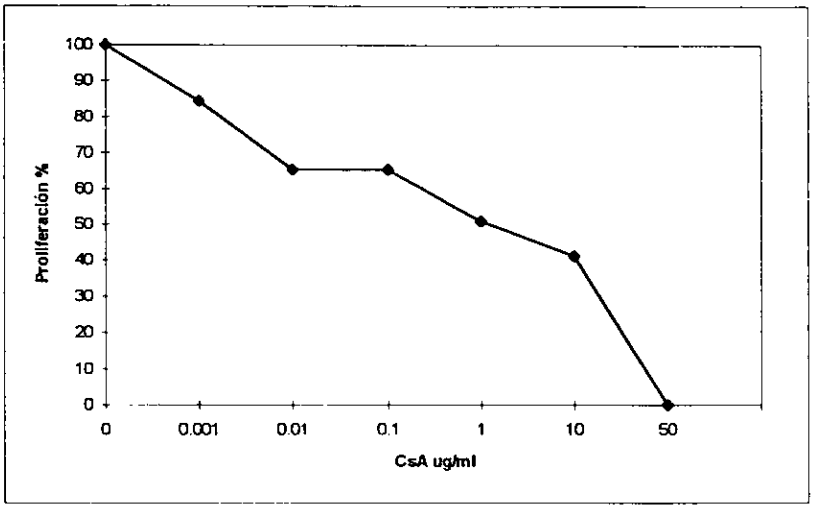


Fig. 22 Inhibición *in vitro* de la proliferación de trofozoítos de *E. histolytica* por CsA. Trofozoítos de 24 h de crecimiento se incubaron con las diferentes concentraciones de CsA indicadas y al término de 72 h se contaron en cámara de Neubauer. La IC_{50} se observó entre 1 y 10 μg/ml de CsA; Dósis de 50 μg/ml ó mas inhibieron totalmente la replicación.

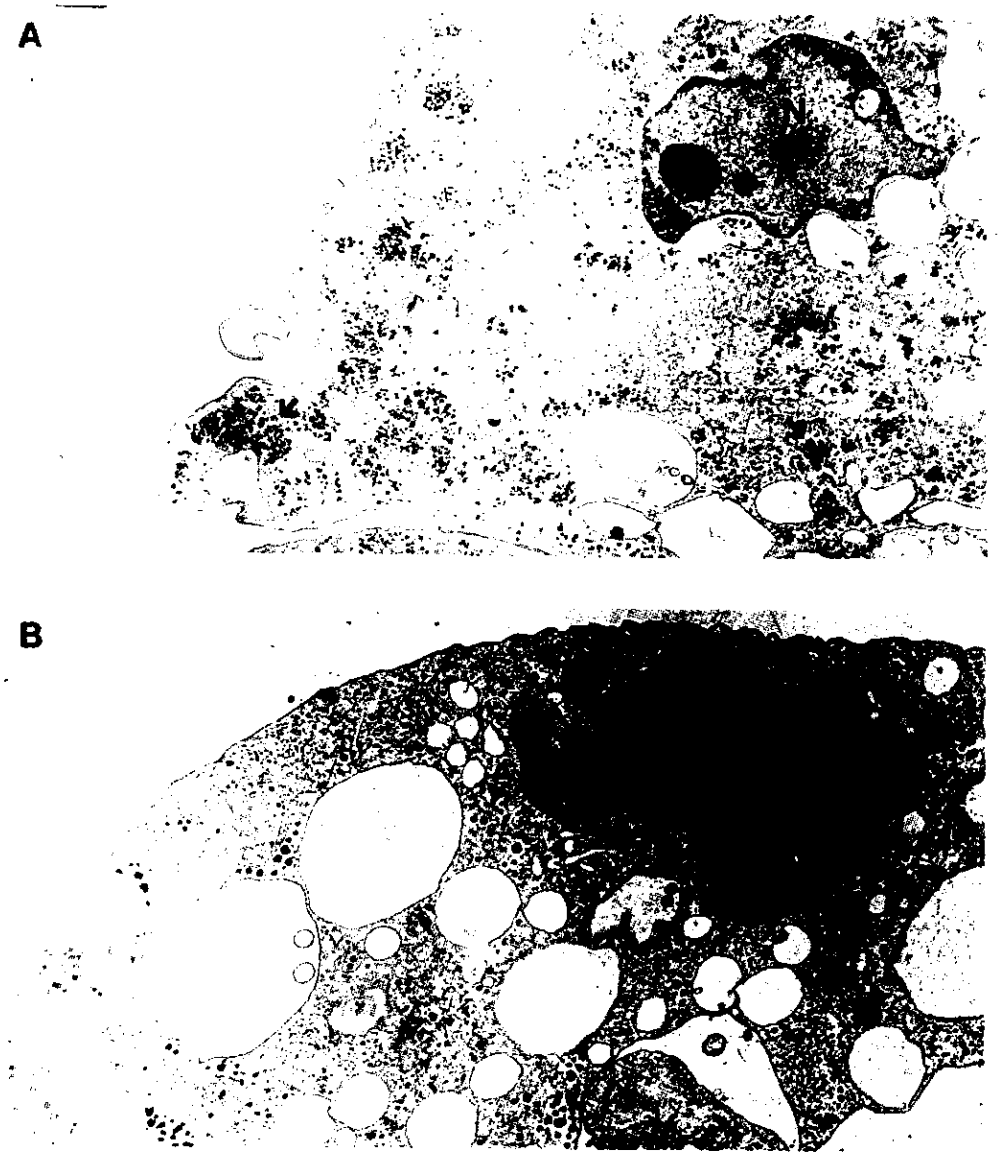


Fig. 23) Microscopía electrónica de transmisión de un trofozoíto de *E. histolytica* tratado con 50 µg/ml de CsA por 48 h (A) y de un trofozoíto no tratado (B). Note la gran cantidad de gránulos densos, algunos en cúmulos, presentes en la mayoría del citoplasma del trofozoíto tratados con la droga (algunos pocos señalados por flechas). N: núcleo.

Con la finalidad de determinar si la CsA indujo la muerte de los trofozoítos por apoptosis, además de analizar la integridad del núcleo por microscopía electrónica, se aisló el DNA de los trofozoítos tratados con 50 µg/ml de CsA para evaluar su fragmentación, una característica típica de la muerte apoptótica. Se observó que el DNA aislado de los trofozoítos tratados se resolvió en gel de agarosa como una banda sencilla de peso molecular elevado idéntica al DNA de los trofozoítos no tratados (Fig. 24). Estos resultados sugieren que la CsA mata a las amibas por una vía diferente de la muerte por apoptosis y que probablemente esté relacionado con el efecto que se le ha descrito a la CsA sobre los linfocitos T.

Efecto de la CsA sobre el desarrollo del AHA. Bajo las condiciones en que se administró la droga en este ensayo *in vivo*, la CsA exacerbó en el 100% de los casos el desarrollo de los abscesos hepáticos amibianos. Se observó cierta correlación directa entre el grado de exacerbación y la concentración a la cual se administró la droga (Figs. 25B, C y D). Así, aun cuando el absceso amibiano se extendió por toda la superficie del hígado, el peso del absceso fue mayor en el caso de los animales tratados con mayor concentración de CsA. En el caso de los animales no tratados con CsA, el absceso hepático se mostró más localizado y menos intenso (Fig. 25A). A pesar de tratarse de resultados poco alentadores, estos están de acuerdo con aquéllos que se han obtenido en la mayoría de los ensayos de protección *in vivo* con CsA sobre agentes infecciosos, a excepción de *Plasmodium* y *Leishmania* (Chappell y Wastling, 1992). Sin embargo, para poder ser más concluyentes al respecto, creemos que se deben realizar una serie de pruebas que abarquen variaciones relacionadas principalmente con la vía, la dosis y el momento de administración de la droga.

Fig. 24 Electroforesis en gel de agarosa de DNA extraído de trofozoítos de *E. histolytica* tratados (A) y no tratados (B) con CsA. El DNA se extrajo de trofozoítos tratados durante 48 h con 50 μg/ml de CsA. PM: marcadores de pesos moleculares.

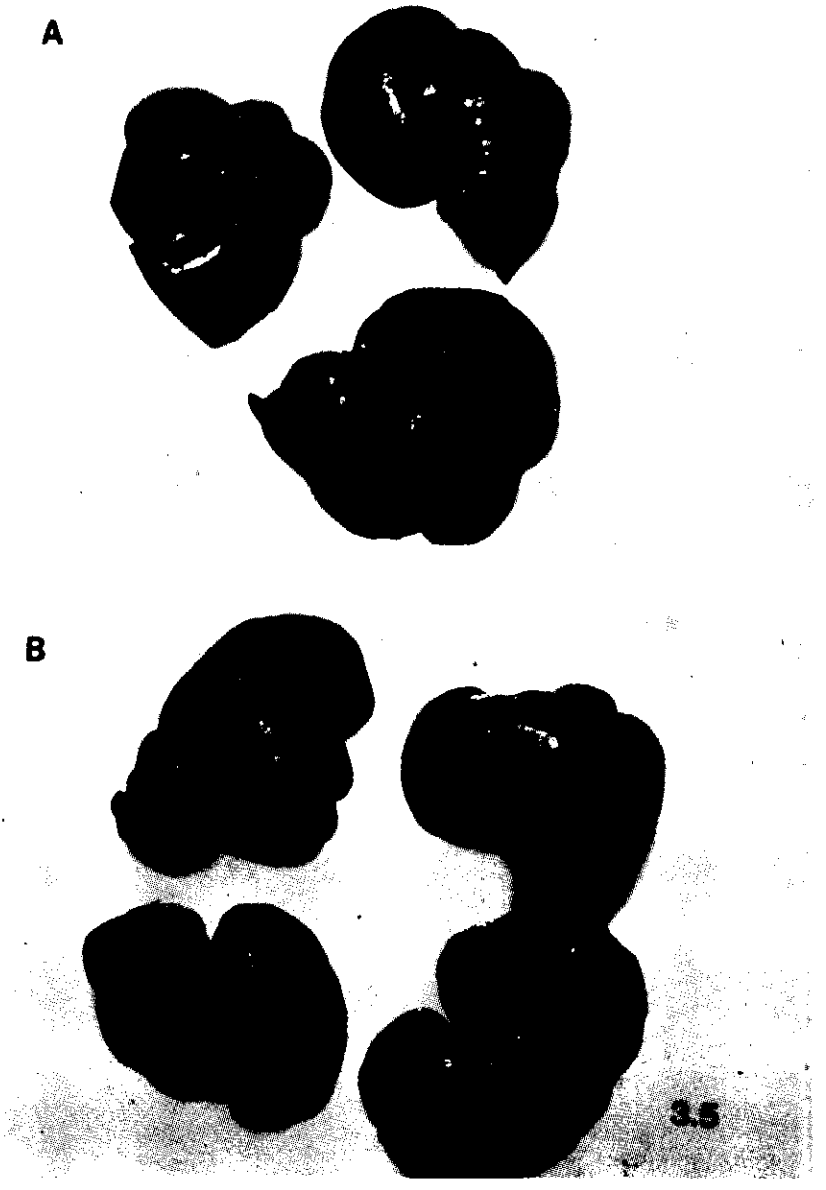
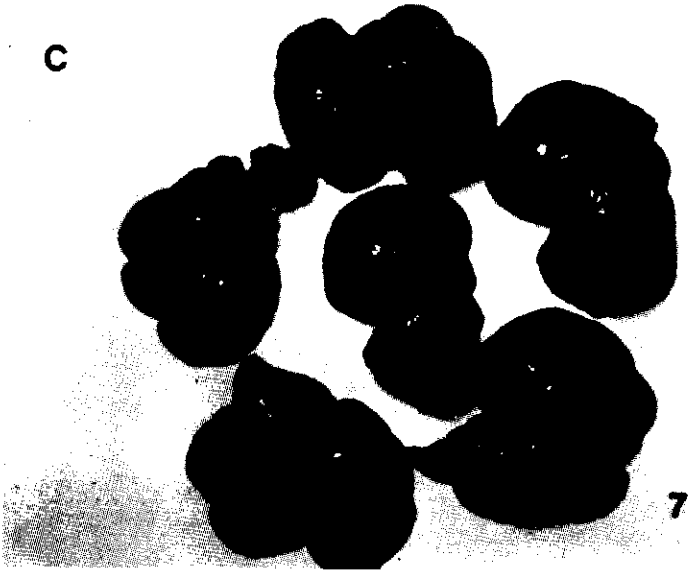


Fig. 25 Efecto del tratamiento de CsA sobre el desarrollo de abscesos hepáticos amibianos (AHA) en hámsters. A) AHA en hámsters no tratados con CsA. B) AHA en hámsters tratados con 3.5 mg de CsA /kg de peso. C) AHA en hámsters tratados con 7 mg de CsA/kg de peso. D) AHA en hámsters tratados con 15 mg de CsA/kg de peso.

C



D

DISCUSION

En este trabajo, se aislaron y caracterizaron clonas de cDNA de *E. histolytica* aprovechando la respuesta de IgAs en pacientes humanos y en un portador asintomático. Los anticuerpos IgAs presentes en altas concentraciones en las secreciones externas, son la primera defensa específica contra las infecciones naturales, en particular contra aquéllas que penetran por alguna vía asociada al sistema inmunitario de mucosas, como es la entérica. Se ha demostrado que los anticuerpos IgAs son protectores contra algunas infecciones de origen viral, bacteriano y parasitario (Morisaki y col., 1983; Tse y Chadee, 1991; Roach y col., 1991). En el caso de la amibiasis el papel biológico de los anticuerpos IgAs es prácticamente desconocido. Sin embargo, existen una serie de evidencias que sugieren un posible papel protector para las IgAs. Por ejemplo, se ha demostrado una correlación positiva entre la presencia de los anticuerpos IgAs y una baja incidencia de la amibiasis en niños de áreas endémicas, durante el tiempo en que son alimentados con leche materna. Unas vez que las madres suspendieron el proceso de amamantamiento, los niños comenzaron a infectarse y presentar las manifestaciones clásicas de la amibiasis intestinal (Islam y col., 1988). De igual forma, nuestro grupo reportó que los anticuerpos IgAs de la saliva de pacientes con amibiasis intestinal son capaces de bloquear la adherencia de los trofozoítos a células de mamífero (Carrero y col., 1994; Kelsall y Ravdin,1995). Ya que la adherencia es considerada por muchos como un paso esencial para la destrucción de tejido por *E. histolytica* (Ravdin, 1986), los resultados que reportamos apuntan a un posible papel protector de los anticuerpos IgAs contra la amibiasis intestinal. Sin embargo, las evidencias son aún preliminares.

Existen una serie de razones que apoyan la posibilidad de obtener una vacuna contra la amibiasis. Entre ellas se cita la relativa sencillez de su ciclo de vida con dos estadios del parásito (trofozoíto y quiste), ausencia de hospedero intermediario, de vectores y de reservorios (Barker y Swales, 1972); la escasa reincidencia de la infección invasora en

pacientes convalecientes del AHA (De León, 1970; Knobloch y Mannweiler, 1983) y finalmente los resultados de protección en modelos experimentales con antígeno total (Meerovitch y Chadee, 1988), fracciones membranales y lisosomales (Begovich, 1978; Sepúlveda 1980), proteínas purificadas(Petri y Ravdin, 1991; Ravdin y col., 1993) y más recientemente con proteínas recombinantes (Zhang y col., 1994; Zhang y Stanley Jr, 1994). Siendo la amibiasis una enfermedad que se inicia por vía entérica y la respuesta inmune secretoria el mecanismo específico de defensa local, nosotros pensamos que una posibilidad para el desarrollo de una vacuna es el uso de antígenos del parásito capaces de inducir una respuesta local de tipo IgA en humanos. En ese sentido, a pesar de que existen reportes que demuestran el desarrollo de una respuesta inmune secretoria en la amibiasis intestinal y el reconocimiento de ciertos antígenos amibianos (denotados por su peso molecular) por los anticuerpos IgAs, se desconoce sin embargo la naturaleza de esas proteínas, con la excepción de la lectina con afinidad por galactosa (Kelsall y col., 1994).

En este trabajo, se aislaron 18 clonas de cDNA de las cuales se caracterizaron total o parcialmente 10 antígenos de *E. histolytica* reconocidos por anticuerpos IgAs. A diferencia de los resultados de reconocimiento de antígenos por immunotransferencia con las muestras de salivas, en el cual se reconocen una amplia variedad de proteínas amibianas, por inmuno-tamizaje se clonaron relativamente pocas. A pesar de que las salivas se absorbieron intensamente con lisado de bacterias para evitar el fondo de reconocimiento, es posible, que con base a la cercanía filogenética y estructural de las amibas con los organismos procariotes (Bakker-Grunwald y Wöstmann. 1993), una buena parte de los anticuerpos IgAs anti-amiba fueran absorbidos por reacción cruzada con las proteínas bacterianas. La determinación de la identidad de las clonas aisladas, indica que la respuesta inmune secretoria está dirigida en gran parte a proteínas intracelulares de *E. histolytica*, ya que al menos 7 de los antígenos identificados son citoplásmicos: deshidrogenasa de acetaldehído/alcohol, HSP70, enolasa, proteinasa de cisteína, factor 1-α de elongación y dos antígenos no reportados previamente (ciclofilina y proteína ribosomal L-23a). Este reconocimiento de componentes intracelulares correlaciona con reportes que demostraron la inmunogenicidad de las fracciones ribosomales y lisosomales de la *E. histolytica* (Sepúlveda, 1980), y además su reconocimiento por

anticuerpos IgAs de pacientes con amibiasis intestinal en estudios de inmunolocalización (O'Shea-Alvarez y col., 1990). La orientación de la respuesta inmune del hospedero hacia componentes intracelulares es visto con frecuencia como una forma de evasión del ataque inmune, ya que reconoce antígenos irrelevantes e inaccesibles que pueden ser liberados de los componentes internos de los parásitos que fracasan en la lucha por sobrevivir. Sin embargo, se ha demostrado que antígenos citoplásmicos de las fracciones lisosomal y ribosomal de las amibas confieren inmunidad protectora en animales de experimentación (Begovich, 1978; Sepúlveda 1980) y que pueden ser utilizados debido a su alta inmunogenicidad en pruebas fidedignas de diagnóstico.

Los otros tres antígenos clonados con base a la respuesta local de IgA en humanos podrían corresponder a proteínas de membrana: SREHP, CREHP de 29 kDa y EHSalJC1. Los dos primeros, identificados con una mezcla de saliva de pacientes con amibiasis intestinal, han sido descritos previamente como potentes inmunógenos. La SREHP es junto con la lectina con afinidad por galactosa, las proteínas más estudiadas de *E. histolytica*. Esta proteína, presente en la superficie de los trofozoítos, se ha utilizado con éxito en ensayos de protección sobre el desarrollo de absceso hepáticos en hámsters, bien sea como proteína nativa o como proteína de fusión (Zhang y col., 1994). La SREHP se ha probado con éxito como inmunógeno oral en roedores mediante la expresión de una proteína de fusión basada en uno de los dodecapéptidos en una cepa atenuada de *Salmonella tiphimurium* y como proteína de fusión a la subunidad β de la toxina del cólera (Cieslak y col., 1993; Zhang y col., 1995). Recientemente, el mismo grupo logró obtener protección contra el desarrollo de absceso hepático amibiano en jerbos, por inmunización oral con la cepa de *S. tiphimurium*, expresando altos niveles de SREHP como proteína de fusión a MBP (proteína de unión a maltosa). La inmunización oral resultó en la obtención de altos niveles de anticuerpos IgAs locales y de IgG séricos, sugiriendo que la inmunización oral con la proteína recombinante de SREHP puede generar inmunidad protectora contra la amibiasis invasora (Zhang y Stanley Jr, 1996). A pesar de estas observaciones no se había demostrado que la SREHP fuera reconocida por anticuerpos IgAs de pacientes y que por lo tanto pudiera ser inmunógeno de este tipo de respuesta en humanos. En el presente trabajo se aislaron con la

IgA de saliva, una clona de cDNA que codifica para una isoforma de SREHP, indicando que esta proteína es capaz de generar una respuesta secretora en humanos. La isoforma de la SREHP se presenta con 6 octapéptidos en tandem en lugar de los 4 que se habían reportado previamente (Stanley y col., 1990). Este resultado sugiere que la SREHP forma parte de una familia de proteínas que se diferencian sólo en el número de repeticiones que posee. De igual manera, el grupo de Tannich ha identificado homólogos de esta proteína en *E. dispar* a los cuales denominaron en forma genérica transcritos K2 (Kohler y Tannich, 1993). La identificación de isoformas, obliga a reconsiderar la idea propuesta por Diamond y Clark de utilizar un sencillo PCR basado en SREHP como marcador molecular para identificar aislados y diferenciar *E. histolytica* de *E. dispar*. Así, la presencia de por lo menos dos isoformas de SREHP en la misma especie podría dificultar la interpretación de los resultados del PCR.

En cuanto a la CREHP de 29 kDa, es otra proteína altamente inmunogénica cuya localización en los trofozoítos es controversial (Flores y col., 1993; Bruchhaus y Tannich, 1993). La CREHP se ha identificado como una alkil hidroperóxido reductasa, enzima que inactiva el peróxido de hidrógeno y que por lo tanto puede participar como mecanismo de defensa del parásito contra los radicales libres de oxígeno (Bruchhaus y col., 1997). Esta proteína fue aislada originalmente a través de un tamizaje diferencial con la finalidad de identificar antígenos exclusivos de amibas patógenas, por lo que se sugiere que sólo se expresa en *E. histolytica* y no en *E. dispar* (Torian y col., 1990). La CREHP es un antígeno amibiano altamente inmunodominante para ambos tipos de respuestas inmunes, ya que es capaz de inducir proliferación de PBMC de pacientes con AHA y ser reconocida por más del 80% de sus sueros (Flores y col., 1993; Soong y col., 1995). En ensayos de protección en jerbos sobre el desarrollo de AHA, la CREHP expresada como una proteína de fusión con un pequeño tallo de 6 histidinas sólo logro una protección discreta del 54% (Soong y col., 1995). Sin embargo, se ha sugerido que la escasa protección observada con CREHP se debió a las condiciones en las cuales se realizó el ensayo (Stanley Jr, 1996). Al igual que la SREHP, no se había demostrado que la CREHP fuera reconocida por anticuerpos IgAs y que por lo tanto pudieran ser propuestos como inmunógenos orales en humanos.

Otra proteína altamente inmunogénica de la amiba es la anteriormente mencionada lectina con afinidad por galactosa de 170 kDa. Esta proteína no fue clonada en este estudio; sin embargo, es la única que se había identificado previamente como blanco de los anticuerpos IgA de saliva de pacientes con amibiasis intestinal (Carrero y col., 1994) y de saliva de pacientes con AHA (Kelsall y col., 1994). Esta proteína junto con la SREHP constituyen actualmente los principales candidatos a vacuna contra la amibiasis. Sin embargo, los resultados de protección con la lectina han sido inconsistentes debido a la presencia en su secuencia de epítopes que exacerban la infección. Incluso el bloqueo de alguno de ellos con anticuerpos monoclonales incrementa la adherencia de trofozoítos a monocapas de células de mamíferos en lugar de inhibirla (Petri y col., 1990). Recientemente se dio un paso importante en ese sentido al identificarse en la lectina una región de 25 aminoácidos que genera anticuerpos que protegen por inmunización pasiva contra el AHA en hámsters. Además la presencia de altos títulos de anticuerpos contra esta región de la lectina correlaciona con la protección que se observa en portadores asintomáticos de *E. histolytica* (Lotter y col., 1997).

Como se mencionó anteriormente, los anticuerpos IgAs de humanos permitió el aislamiento de tres clonas de cDNA que codifican para proteínas no reportadas con anterioridad en *E. histolytica*. Dos de ellas aisladas con saliva de un paciente con AHA, codifican para la ciclofilina (EhCyp) y la proteína ribosomal L-23a (EhRP-L12a) de *E. histolytica*. La tercera, aislada con la saliva del portador asintomático, codifica para una proteína desconocida rica en ácido glutámico (EHSalJC1) que carece de homología con aquéllas acumuladas en la base de datos. Debido a que no se sabía nada de la ciclofilinas en la amiba y al hecho de que en otras enfermedades parasitarias se está considerando la posibilidad de utilizar a la CsA o análogos no inmunosupresores de la misma, como drogas anti-parasitarias (Chappell y Wastling, 1992), decidimos caracterizar la EhCyp y determinar el efecto *in vitro* e *in vivo* de la CsA sobre la amiba y la modulación de la infección amibiana. La secuencia completa de la EhCyp muestra que a diferencia de las Cyps de la mayoría de los eucariotes excepto las plantas, ésta carece de una secuencia adicional en el

extremo amino-terminal que usualmente funciona como péptido señal para la localización en un compartimiento subcelular. En base a lo anterior y al hecho de que la amiba es una célula rudimentaria que carece de la mayoría de los compartimientos típicos de las células eucariontes (mitocondria, retículo endoplásmico, aparato de Golgi, etc), sugerimos que EhCyp podría estar presente en el citoplasma de los trofozoítos como una proteína libre o asociada a otras formando complejos en los que funciona como chaperona. Varias observaciones indican que las actividad de PPIasa debida a la ciclofilina y a FKBP son inhibidas específicamente *in vitro* por las drogas que se unen a cada una de ellas: CsA y FK506, respectivamente. La presencia de ciclofilina en extractos citosólicos de trofozoítos de *E. histolytica* se demostró por detección de una típica actividad de PPIasa que es inhibida prácticamente en su totalidad por la adición de CsA. Este resultado junto con la identificación en la secuencia de EhCyp de los aminoácidos esenciales para la unión de la CsA, indica que EhCyp une la droga inmunosupresora CsA y que es responsable de la mayoría de la actividad de PPIasa detectada en la fracción soluble de los trofozoítos.

A pesar de que el efecto antiparasitario de la CsA se conoce desde hace varios años, nada se había hecho al respecto de *E. histolytica*. *In vitro* la CsA inhibe la replicación de la mayoría de los parásitos protozoarios en los que se ha ensayado. Sin embargo *in vivo*, sólo en malaria la actividad antiparasitaria de la CsA es superior a los efectos inmunosupresores causados en el hospedero infectado. En el caso de la leishmaniasis, el efecto protector de la CsA es debido más a una inmunomodulación de la respuesta del hospedero (disminuye el número de células potenciales de ser infectadas) que a un efecto antiparasitario (Chappell y Wastling, 1992). Como la mayoría de los protozoarios, los trofozoítos de *E. histolytica* fueron susceptibles in vitro al tratamiento con la CsA. A mayor concentración de CsA, mayor fue el efecto inhibidor de la replicación del parásito. La IC_{50} determinada para *E. histolytica* (1 a 10 μg/ml) es relativamente similar a la reportada para *Plasmodium vivax* (IC_{50} de 1.7μg/ml) pero difiere considerablemente de aquélla obtenida para *P. falciparum* (IC_{50} de 0.12μg/ml, la más sensible) y para *L. major* (IC50 > 25 μg/ml, la más resistente) (Kocken y col., 1996; Solbach y col., 1986). Se desconoce la razón de la diferencia de susceptibilidades a la CsA, pero ésta podría estar asociada a la capacidad del parásito de

tomar o eliminar la droga así como al hecho de que su maquinaria transcripcional pudiera ser afectada en diferentes grados, debido a la presencia o ausencia de una calcineurina o proteína tipo calcineurina en los parásitos. En el primero de los casos, existe evidencias de que la CsA actúa como sustrato para las glicoproteínas P (Pgp) aisladas de células de mamífero, unas proteínas involucradas en la desintoxicación celular de metales y drogas. Este tipo de proteínas han sido identificadas en muchos parásitos incluyendo *E. histolytica*, en el que se han descrito por lo menos 4 genes (genes *EhPgp*) (Orozco y col., 1995).

Desconocemos cómo la CsA ejerce su efecto anti-amiba *in vitro*, pero como ha sido propuesto para otro parásitos y derivado del conocimiento que se tiene del efecto de la CsA sobre los linfocitos T, es posible que ésta se una a EhCyp en el citoplasma formando un complejo que bloquea la actividad de una calcineurina fosfatasa dependiente de Ca^{2+} y calmodulina. Esta inhibición puede prevenir la desfosforilación y consiguiente translocación de factores nucleares de la familia NF-AT involucrados en la transcripción de genes que codifican para factores de replicación. Otra posibilidad está asociada a la inactivación de la actividad de PPIasa por la CsA. Esta actividad, detectada en el extracto soluble de los trofozoítos, se ha sugerido que es importante como chaperona en el correcto plegamiento de enzimas metabólicas, factores de transcripción y proteínas en general que los hace funcionalmente activos. En cuanto al efecto *in vivo* es claro que bajo las condiciones en que se realizó el ensayo, la actividad inmunosupresora de la CsA pesó mas que su efecto anti-amiba. A pesar de este resultado, que es similar al que se ha obtenido en la mayoría de ensayos de protección *in vivo* contra diferentes agentes infecciosos, no se puede descartar la posibilidad de obtener resultados positivos mediante la modificación del ensayo de protección (vía de administración y tiempo y duración de la misma) o mediante el uso de drogas análogas con poco o ningún efecto inmunosupresor.

Con respecto a EHSalJC1, la única clona aislada con la saliva de un portador asintomático, parece ser parte de una familia de proteínas nuevas identificadas en *E. histolytica* (detección de por lo menos 4 transcritos en el análisis de Northern blot). Es de interés mencionar que un antígeno nuevo de 39 kDa (K18), rico en ácido glutámico como

EHSaIJC1, fue aislado de una biblioteca de cDNA de la cepa de *E. histolytica* SFL-3 usando un antisuero producido contra una fracción de membranas seguido por reconocimiento con sueros de pacientes con amibiasis invasora (Plaimauer y col., 1994). El gen codificante de la proteína K18 contiene un intrón y es ligeramente diferente entre *E. histolytica* y *E. dispar*. Aunque EHSaIJC1 y K18 son proteínas antigénicas y ricas en ácido glutámico son diferentes como se determinó por análisis comparativo de las secuencias. Sin embargo, también es posible que la diferencia se deba a que son proteínas aisladas de diferentes cepas de *E. histolytica*. Son necesarios estudios adicionales para definir el papel de esta proteína en la fisiología de la amiba, así como en la infección por el parásito.

El análisis de las clonas aisladas con las diferentes muestras de saliva, así como el reconocimiento de los péptidos recombinantes por inmunoelectrotransferencia (CREHP no fue reconocido por la saliva del portador asintomático y EHSaIJC1 no fue reconocido por la saliva de los pacientes con amibisis intestinal), sugieren que no hay reacción cruzada entre las muestras de saliva. Esta observación abre la posibilidad de que los pacientes con infección entérica y los portadores asintomáticos reconozcan diferentes antígenos del parásito y que éstos pudieran estar relacionados con susceptibilidad o resistencia a la infección. Por el contrario, la saliva del paciente con AHA reconoce un espectro mucho más amplio de antígenos, inclusive ambas proteínas recombinantes. La escasa reincidencia de la amibiasis invasora en estos pacientes convalecientes podría deberse, al menos en parte, a esa capacidad de reconocimiento de la amiba.

A través de los patrones de zimodemas, los cuales se han usado para diferenciar *E. histolytica* de *E. dispar*, se ha logrado demostrar que a diferencia de lo que antes se pensaba, existen individuos portadores asintomáticos que pueden estar infectados *con E. histolytica*(Gathiram y Jackson, 1987). Aunque el portador asintomático usado en el presente estudio no se caracterizó en cuanto al tipo de amiba que portaba, hay que tomar en cuenta que EHSaIJC1 se aisló de una biblioteca de *E. histolytica* y que la saliva de los pacientes con amibiais intestinal y por lo tanto infectados con amibas patógenas no lo reconocieron. También se ha propuesto que la susceptibilidad a la amibiasis invasora puede

estar asociada con cierto fondo genético (HLA-DR3 y complotipo SCO1) (Arellano y col., 1991). La caracterización del reconocimiento diferencial entre pacientes y portadores asintomáticos, podría proveer información útil para la identificación de antígenos candidatos para el desarrollo de una vacuna y diagnosis.

Un ensayo inicial de protección se llevó a cabo en ratones de la cepa susceptible C3H/HeJ por inmunización oral con los péptidos recombinantes de CREHP y EHSaIJC1. De acuerdo con reportes previos de nuestro laboratorio, la inoculación intracecal de trofozoítos recuperados después de varios pases por hígado de hámster, produjo numerosas lesiones sobre la superficie del epitelio que se asemejan a aquellas que se producen en humanos (Ghosh y col., 1994). Los trofozoítos sobreviven en el lumen cecal de los ratones por más de 20 días generando una enfermedad leve que puede ser evaluada por la apariencia del ciego y su contenido, la presencia de trofozoítos en heces y por examinación microscópica de secciones de tejido cecal. Así, los ratones C3H/HeJ constituyen un modelo útil para investigar muchos aspectos de la infección intestinal, incluyendo la evaluación de candidatos a vacuna. El análisis de las secciones de tejido cecal mostró que los ratones no inmunizados tenían numerosas y extensivas lesiones sobre el epitelio superficial con abundante infiltrado inflamatorio y trofozoítos invasores en la submucosa y lámina propia. Por el contrario, la mayoría de los ratones inmunizados con la CREHP de 29 kDa no mostraron daño sobre la superficie del epitelio, ni infiltrados con trofozoítos. Los ratones inmunizados con EHSaIJC1 mostraron ligeros infiltrados, más pequeños y menos numerosos que los observados en los animales no inmunizados, además de carecer de trofozoítos invasores. A diferencia de los no inmunizados, ninguno de los ratones pertenecientes a ambos grupos inmunizados liberaban trofozoítos con las heces en los tiempos cercanos al día de sacrificio.

Con base en la conjunción de la apariencia externa de los ciegos, consistencia del contenido cecal y las obervaciones microscópicas de las lesiones del ciego, concluimos que bajo nuestras condiciones experimentales la inmunización oral con el péptido recombinante de la CREHP de 29 kDa confirió una muy buena protección contra la infección intestinal en

el ratón. En el caso de EHSaIJC1, la presencia de algunas pequeñas lesiones en los ciegos así como de ligeros infiltrados sugieren que la protección fue sólo parcial. En ambos casos la protección parece estar asociada con la producción de altos títulos de anticuerpos IgAs anti-amiba detectados sólo en las heces de los ratones inmunizados antes del reto intracecal con los trofozoítos (Fig. 17). Sin embargo, en los ratones inmunizados también se desarrolló una elevada respuesta sérica antiamiba, principalmente del isotipo IgG, que deja abierta la posibilidad de que ambas respuestas humorales, secretora y sérica, sean importantes en la adquisición de la protección. En ese sentido, existen reportes que sugieren un papel importante para los anticuerpos séricos en la protección del hospedero a la invasión por *E. histolytica*, basados en una asociación entre la presencia de una respuesta sérica en humanos y la baja incidencia de amibiasis, así como de observaciones que demuestran que la transferencia pasiva de anticuerpos de pacientes con AHA a animales de experimentación susceptibles (ratones SCID) los protege contra el reto hepático con trofozoítos (Choudhuri y col., 1991; Seydel y col., 1996). Ya que la amiba no logra aparentemente sobrevivir ni penetrar el tejido intestinal, el mecanismo efector de la protección debe ser local.

La importancia de una respuesta sérica puede radicar en la contribución de células plasmáticas productoras de anticuerpos del torrente sanguíneo a la placa intestinal, por migración a través de las vías linfáticas. En cuanto a la inmunidad mediada por células, es posible que en el caso de la infección intestinal de los ratones no desempeñe un papel importante en la protección observada, debido a que como se demostró anteriormente (Ghosh y col., 1995) y como lo corroboramos en nuestros ensayos, la proliferación de células T determinada en ensayos de incorporación de timidina tritiada (dato no mostrado), se encuentra totalmente suprimida Se desconoce la forma en que la amiba suprime al sistema inmune del ratón, pero se ha sugerido que es a través de la inhibición de la transducción de señales de la superficie. La presencia de grandes infiltrados en los cortes de ciego de los ratones no inmunizados, parecen estar asociados más a daño local que a una respuesta protectora. Estos infiltrados, principalmente de polimorfonucleares y linfocitos, fueron muy grandes alrededor de amibas escasas, aparentemente intactas, que lograron atravesar el

epitelio intestinal, mientras que en los ratones inmunizados (principalmente con CREHP) no se observaron ó fueron pequeños, sin daño aparente de la mucosa intestinal.

Los hallazgos más interesantes que apoya el objetivo planteado en esta tesis lo constituyen las recientes observaciones de los grupos de Stanley y de Mann, quienes de manera independiente lograron obtener protección contra el AHA en jerbos por inmunización oral con una cepa atenuada de *S. typhimurium* que expresa en el caso de Stanley, altos niveles de un fragmento de la proteína SREHP (Zhang y Stanley, 1996) y en el caso de Mann, altos niveles de una porción de la lectina de 170 kDa (Mann y col., 1997). Al igual que los hallazgos presentados aquí, ellos obtuvieron altos niveles de anticuerpos IgAs e IgG séricos anti-amiba que están íntimamente asociados con el nivel de protección. Estos hallazgos junto con nuestras observaciones, apoyan la idea que se plantea en esta tesis de que antígenos amibianos pueden ser utilizados como inmunógenos orales para inducir respuestas inmunes protectoras que constituyan las bases para el desarrollo de una vacuna práctica contra la amibiasis intestinal. El uso de las nuevas tecnologías del DNA recombinante para la obtención *in vitro* de las proteínas, así como la disponibilidad del modelo de amibiasis intestinal en ratones, facilitan las pruebas para detectar los candidatos más óptimos en el desarrollo de esta vacuna.

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Human Secretory Immunoglobulin A Anti-*Entamoeba histolytica* Antibodies Inhibit Adherence of Amebae to MDCK Cells

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The presence of secretory immunoglobulin A (IgA) anti-*Entamoeba histolytica* antibodies in the saliva of patients with intestinal amebiasis was demonstrated by immunoblot assay, and the capacity of these antibodies to inhibit amebic adherence to a monolayer of MDCK cells was analyzed. Inhibition was due to IgA antiamebic antibodies and in part to anti-Gal-binding-lectin antibodies, as demonstrated by absorption experiments with total amebic extract and with the fraction of Gal-binding lectin. These results emphasize the relevance of secretory IgA antibodies in the phenomenon of *E. histolytica* adherence to epithelial cells.

Amebiasis due to *Entamoeba histolytica* is an infectious disease of worldwide distribution which affects 500 million people and is responsible for approximately 50 million cases of tissue invasion and approximately 60,000 deaths per year (22). In many developing countries, amebiasis is one of the main medical problems, affecting all socioeconomic levels of the population (6).

Tissue damage by *E. histolytica* is contact dependent, resulting in lysis of the cells to which amebae adhere (3, 18). The molecules which mediate adhesion are lectins located on the amebic surface (17), of which several have been described previously (1, 15, 19). Among them, an amebic lectin with affinity for galactose (Gal) has been proposed as the main molecule responsible for the adhesion of trophozoites to colonic mucin and epithelial cells (3, 13).

The role of secretory immunoglobulin A (IgA) antibodies against surface molecules of *E. histolytica*, including the Gal-binding lectin that participates in adherence, has not been studied in depth. It is believed that the secretory immune response against amebic surface molecules plays an important role when it comes to parasite colonization of the intestine, since one of the protective mechanisms mediated by IgA is the inhibition of microorganism adherence to and colonization of this tissue (9, 10). It was therefore interesting to determine whether anti-*E. histolytica* IgA antibodies and specifically those to the Gal-binding lectin are present in the saliva of patients with amebiasis and to determine whether these antibodies are able to inhibit adherence of amebae to MDCK cells in vitro.

Axenic cultures of *E. histolytica*, HM1:IMSS strain, were grown in TYI-S-33 medium with 100 U of penicillin per ml–100 μg of streptomycin sulfate per ml–0.25 μg of amphotericin B per ml at 37°C in culture flasks (5). Trophozoites were harvested after 72 h of growth by chilling the flasks on ice for 10 min and centrifuging at 150 × *g* for 10 min at 4°C. The parasites were then washed three times with phosphate-buffered saline (PBS), pH 7.4, by centrifugation as described above. The viability of parasites was assayed by exclusion of trypan blue, and it was about 91%.

MDCK cells were grown in Dulbecco's modified Eagle medium (GIBCO Laboratories, Life Technologies, Grand Island, N.Y.) at pH 6.8, supplemented with 5% fetal bovine serum–100 U of penicillin per ml–100 μg of streptomycin per ml–2.4% sodium bicarbonate, in culture flasks at 37°C and in a humid atmosphere with 5% CO_2. Monolayers of MDCK cells were obtained in 96-well culture plates (50,000 cells per well). The monolayer was washed with 0.15 M NaCl and fixed with 0.25% glutaraldehyde for 10 min. Then, the plates were washed with a solution of 0.1 M glycine and finally with 0.15 M NaCl and stored at 4°C until used (8).

Optimal conditions for adherence of amebae to an MDCK cell monolayer were obtained with 50,000 amebae per microwell, in 2 mM PBS, pH 5.7, by coincubating them for 15 min at 37°C with gentle shaking (75 rpm). These conditions were applied to all experiments reported herein. The trophozoites adhering to the cells were then fixed with 0.25% glutaraldehyde in 0.15 M NaCl for 10 min at room temperature. The next step was to block the plates with 200 μl of 1% bovine serum albumin for 1 h at room temperature. The plates were washed with 0.05% Tween 20–1% BSA in PBS, pH 7.4 (T-BSA-PBS). Subsequently, 50 μl of biotinylated monoclonal anti-*E. histolytica* antibody to a surface molecule of *E. histolytica* (11) diluted 1:400 was added and the plate was incubated for 1 h at 37°C. The plates were then washed as described above. A streptavidin-peroxidase conjugate (1:2,000) was added and incubated for 1 h at 37°C. Then, the plates were washed again as described above. Finally, *o*-phenylenediamine was used as a substrate; the reaction was stopped with 2.5 N H_2SO_4 and measured at 490 nm in an enzyme-linked immunosorbent assay (ELISA)-Processor M. A positive correlation was found between the number of amebae binding to the MDCK cells and optical density at 490 nm.

Saliva was collected from 24 patients with a diagnosis of intestinal amebiasis confirmed by stool examination and by salivary IgA anti-*E. histolytica* antibody detection (4). These saliva samples were kindly provided by Edmundo Godínez Camacho from the Hospital General de México. Control samples were obtained from 16 healthy individuals with no clinical symptoms of amebiasis and from 9 patients with nonamebic parasitic diseases. For both control groups, stool examination (three samples, obtained on three consecutive days) and salivary anti-*E. histolytica* antibody assay were performed; they were negative, indicating the absence of amebae or their antibody, respectively. However, the control group of patients with other parasitic diseases yielded at least one of the following parasites: *Hymenolepis nana*, *Entamoeba coli*, *Giar-*

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dia lamblia, *Trichuris trichiura*, *Enterobius vermicularis*, *Chilomastix mesnili*, and *Ascaris lumbricoides*. The saliva samples were centrifuged at 10,000 × *g* for 30 min, and the supernatant was frozen at −20°C until used.

Amebae (50,000 parasites) were preincubated with 1 ml of saliva from patients or controls previously diluted 1:1 with a mucolytic agent (Mucosil-20; Dey Laboratories Inc., Napa, Calif.) for 1 h at 37°C. These immunoglobulin-treated trophozoites were washed and used in the adherence ELISA described above. For all saliva samples, a control of possible interference due to competition between the monoclonal antibody and IgA from the saliva was run in parallel in the following way: after amebae were fixed, 200 μl of saliva was added and incubated for 1 h at 37°C; afterwards, the plates were washed; and finally, the biotinylated monoclonal anti-*E. histolytica* antibody was added. The reading was subtracted from the ELISA results.

IgA from the saliva samples was purified by affinity chromatography on a jacalin-agarose column (Sigma Chemical Co., St. Louis, Mo.) (2). Fractions of 1 ml were collected, and the protein content was determined at 280 nm in a spectrophotometer. The purified IgA was analyzed by polyacrylamide gel electrophoresis (PAGE) under reducing conditions (sodium dodecyl sulfate [SDS]-PAGE). The characteristic three bands of the secretory IgA were observed: 70, 56, and 23 kDa (12). One hundred fifty micrograms of purified IgA was used in the inhibition assay described above; this amount was the average value of purified IgA from 1 ml of patient's saliva.

Saliva samples were preabsorbed with 150 μg of the Gal-binding lectin or with 150 μg of a total amebic extract for 2 h at 37°C. This amount of antigen was previously known to abolish the reactivity of the saliva IgA with either the total amebic extract or Gal-binding lectin by immunoblot. Then, the saliva samples were used in the adherence ELISA.

The purification of the Gal-binding lectin was performed as reported previously (15). In brief, 5 ml of immobilized D-Gal (Pierce, Rockford, Ill.) was incubated with 20 ml of a 10-times-concentrated supernatant culture medium from amebae (previously dialyzed against 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]–0.14 M NaCl [pH 7.5] for 18 h at 4°C) with gentle agitation. Then the column was washed with HEPES-NaCl. The bound proteins were eluted with 0.8 M Gal, and protein was detected at 280 nm. Positive fractions were analyzed by SDS-PAGE. A Western blot (immunoblot) of the Gal-binding lectin with either a polyclonal anti-*E. histolytica* antiserum (11) or the 3F4 monoclonal anti-Gal-binding-lectin antibody (kindly provided by W. A. Petri, School of Medicine, University of Virginia, Charlottesville) was also performed.

Trophozoites (3 × 10^6) were metabolically labeled with 600 μCi of [^{35}S]methionine (specific activity, 1,200 Ci/mmol) in TYI-S-33 medium without cysteine for 12 h at 37°C. Then, the parasites were harvested by being chilled on ice for 10 min and centrifuged at 150 × *g* for 5 min at 4°C, washed with PBS containing 2 mM phenylmethylsulfonyl fluoride and 1 mM *p*-hydroxymercuribenzoate, and finally lysed by freezing and thawing. The labeled supernatant and the parasites were applied to the immobilized D-Gal column.

Both the total *E. histolytica* extract (800 μg) and the Gal-binding lectin (100 μg) were electrophoresed on SDS–10% polyacrylamide gels and transferred to nitrocellulose paper as described by Towbin et al. (20). Saliva samples from patients and controls were diluted (1:8) in 0.3% Tween–PBS and incubated with the paper strips for 2 h at room temperature. After extensive washing with T-PBS, a goat anti-human IgA antibody conjugated to peroxidase (1:2,000) (Zymed Laboratories, Inc., San Francisco, Calif.) was added. Finally 3',3-diaminobenzidine was used as a substrate.

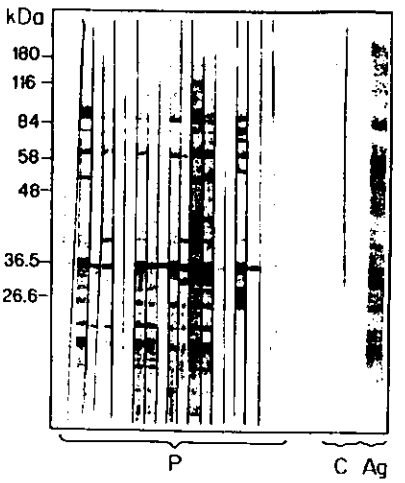

FIG. 1. Immunoblot of amebic antigens recognized by secretory IgA antibodies in saliva (1:8 dilution) of patients with intestinal amebiasis. P, patients with intestinal amebiasis; C, healthy controls; Ag, control of total amebic antigen stained with Coomassie blue.

The results indicate that 85% of the saliva samples from patients recognized various amebic antigens when a total amebic extract was used. Thus, IgA antibodies from patient saliva recognized proteins of 36 kDa (84%), 23 kDa (79%), 59 kDa (68%), 87 kDa (58%), and 26 kDa (50%) (Fig. 1). In addition, 42% of the same samples presented specific IgA antibodies against a protein of 170 kDa. On the other hand, saliva from normal individuals was generally negative and only occasionally showed some 21-, 23-, and 109-kDa bands.

An attempt to purify the lectin of 260 kDa from the supernatant culture medium with affinity to Gal was done by affinity chromatography in an immobilized D-Gal column. A single protein peak was obtained. SDS-PAGE under nonreducing conditions of the pooled peak showed only a band of 260 kDa (Fig. 2, lane B). On the other hand, SDS-PAGE performed under reducing conditions showed four bands of 170, 100, 68, and 30 kDa (Fig. 2, lane C). The bands of 170 and 30 kDa seem to correspond to the molecular mass previously reported for the Gal-binding lectin. Similar results were obtained when the parasites were metabolically labeled with [^{35}S]methionine (Fig. 2, lane D). When this fraction was tested by immunoblot with a hyperimmune anti-*E. histolytica* antiserum, the same four bands were recognized (Fig. 2, lane E). Furthermore, three (170, 68, and 30 kDa) of the four above-mentioned bands were recognized when tested with the 3F4 monoclonal anti-Gal-binding-lectin antibody, which recognizes the 170-kDa subunit epitope I of the Gal-binding lectin (14, 16) (Fig. 2, lane F). When this fraction was used as antigen in an immunoblot assay with the saliva described above, 61% of the samples showed a positive reaction with the protein of 170 kDa (Fig. 3). The bands of 68 and 30 kDa were also recognized by 52 and 43% of the saliva samples, respectively. However, the 100-kDa band was not recognized by any of the patient saliva samples. In contrast, saliva from the healthy groups did not show reactivity with the Gal-binding lectin (Fig. 3).

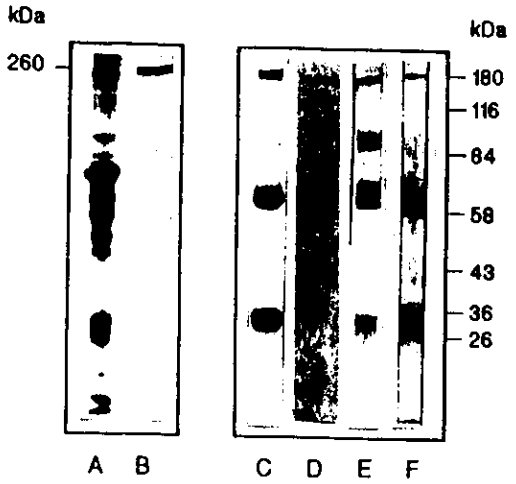

FIG. 2. Purification of lectin by affinity chromatography in an immobilized D-Gal column. The proteins were eluted, pooled, and analyzed by SDS-PAGE. Lanes: A, SDS-PAGE pattern of supernatant applied to column; B, purified fraction analyzed by SDS–7.5% PAGE under nonreducing conditions and stained with Coomassie blue; C, purified fraction analyzed by SDS–10% PAGE under reducing conditions and stained with Coomassie blue; D, purified fraction obtained from parasite metabolically labeled with [^{35}S]methionine; E, purified fraction analyzed by immunoblot with a hyperimmune anti-*E. histolytica* antiserum; F, immunoblot assay with 3F4 monoclonal anti-Gal-binding-lectin antibody.

Considering that the phenomenon of adherence may be mediated by adhesins or lectins and particularly by those with affinity for galactose, we decided to test whether IgA antibodies to amebic proteins and particularly to the Gal-binding lectin could block the adherence of trophozoites to MDCK cells. For this purpose, 24 saliva samples of patients with intestinal amebiasis were tested for inhibition of amebic adherence to a monolayer of MDCK cells. Amebic adhesion was monitored by the ELISA described above. Eighty-five percent of the saliva samples from patients with amebiasis significantly ($P < 0.01$) inhibited the adherence of trophozoites to MDCK cells by more than 20% (mean ± standard error [SE] = 35% ± 2.6%). Saliva samples from patients with other parasitic diseases (13.5% ± 2.5%) and from healthy people (9% ± 1.4%) inhibited adhesion more weakly. The adhesion values of the last two groups are significantly ($P < 0.01$) lower than those for saliva from patients with amebiasis.

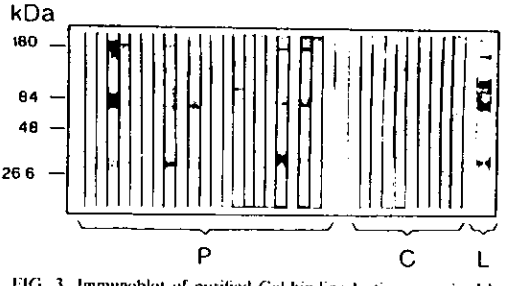

FIG. 3. Immunoblot of purified Gal-binding lectin recognized by secretory IgA antibodies in saliva (1:8) of patients with intestinal amebiasis. P, patients with intestinal amebiasis; C, healthy controls; L, Gal-binding lectin obtained from the immobilized D-Gal column.

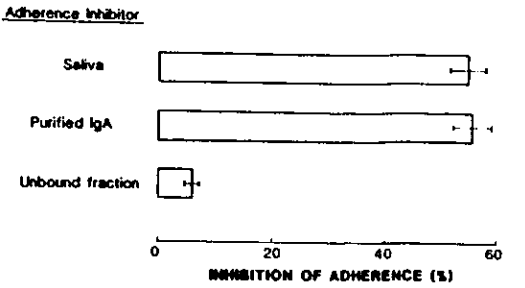

FIG. 4. Inhibition of adherence by patient's saliva and by its purified IgA antibodies. Amebic trophozoites (50,000) were incubated with 150 μg of pure IgA obtained from 1 ml of patient's saliva or with 1 ml of total saliva for 1 h at 37°C and added to an MDCK cell monolayer. The immunoenzymatic assay was monitored as previously described. Both total saliva and pure IgA showed the same inhibitory levels. On the other hand, the unbound fraction obtained during the IgA purification showed a very low inhibitory capacity. Shown are the means ± SEs of four saliva samples.

IgA anti-*E. histolytica* antibodies were purified from four saliva samples by means of minicolumns of jacalin-agarose and examined by SDS-PAGE. Three bands corresponding to the subunits of IgA were observed (data not shown). The pure IgA antibodies were used in the same assay of inhibition of amebic adhesion to MDCK cells as previously described. The results showed that total saliva (1 ml) from amebic patients as well as the IgA purified from them at similar IgA concentrations (150 μg of IgA purified from 1 ml of saliva) inhibited amebic adherence to the same extent (Fig. 4), suggesting that the inhibitory capacity of patient saliva is due to the presence of antiamebic IgA. On the other hand, the material not bound to the same column did not inhibit the amebic adherence to MDCK cells.

An experiment on absorption was conducted to determine whether the inhibition of adherence by antibodies was specific to *E. histolytica* as well as to the Gal-binding lectin. For this purpose, the saliva of five patients was absorbed with total *E. histolytica* extract or with the Gal-binding lectin from the *E. histolytica* trophozoites and then used in the adherence assay. The results showed that the adherence inhibition was reduced by 80% when the saliva samples were previously absorbed with total amebic extract (inhibition mean ± SE = 11.2% ± 3.6%) compared with inhibition with nonabsorbed saliva samples (inhibition mean ± SE = 56.1% ± 2.9%). On the other hand, adherence inhibition was reduced by only 32.7% (inhibition mean ± SE = 37.8% ± 2.7%) when the Gal-binding lectin was used.

The data presented here indicate that saliva from patients with intestinal amebiasis presents secretory antibodies directed against several proteins from an extract of *E. histolytica* and also against the Gal-binding lectin. The Gal-binding lectin obtained in our laboratory when analyzed by SDS-PAGE under nonreducing conditions showed one band of 260 kDa. However, three main bands, 170, 68, and 30 kDa, and a faint band of 100 kDa were observed when analyzed under reducing conditions. When this Gal-binding lectin was transferred to

nitrocellulose paper, the proteins of 170, 68, and 30 kDa were recognized by the 3F4 monoclonal anti-Gal-binding-lectin antibody, suggesting that the lectin was being degraded. One possibility for the proteolytic breakdown observed is that we used a mild protease inhibitor cocktail not containing diisopropyl fluorophosphate, a potent serine protease inhibitor. It was of interest to investigate whether these saliva antibodies inhibit the adhesion of amebae to epithelial cells. For this purpose, we developed an ELISA with MDCK cells and a monoclonal antibody produced against a surface protein of *E. histolytica* (11). The assay showed a direct correlation between the number of adherent amebae and the absorbance reading of the substrate.

Saliva or IgA purified from patients with intestinal amebiasis inhibited adherence of *E. histolytica* to MDCK cells to the same extent (Fig. 4), indicating a direct role of the secretory IgA anti-*E. histolytica* immunoglobulins in the inhibition of adherence phenomenon. The IgA specific to the Gal-binding lectin from *E. histolytica* very likely prevents the attachment of the parasite to the target cell, retaining the microorganisms within the mucus layer and reducing their motility; in addition, they became susceptible to the natural renewal process of the mucus layer (21). Interestingly, saliva obtained from individuals infected with other parasites, including *Entamoeba coli* and *G. lamblia*, did not inhibit amebic adherence to MDCK cells.

The absorption experiments showed that the IgA antibodies to the lectin of 260 kDa with affinity for Gal were in part responsible for the inhibition observed. On the other hand, absorption experiments with the total amebic extract showed that the saliva thus absorbed lost the capacity to inhibit amebic adherence. These results are in agreement with the notion that there must be a group of lectins and adhesins participating in the adhesion of *E. histolytica* to epithelial cells. Several of these lectins have been reported elsewhere (1, 7, 19), but information about their role in the in vivo phenomenon of adherence is limited. It would be of interest to study the secretory response against the other lectins with the adherence ELISA described here.

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Molecular Biology of *Entamoeba histolytica*: A Review[1]

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Abstract

Amebiasis is one of the main causes worldwide of morbidity and mortality by parasites. Application of recombinant DNA technology to the study of *Entamoeba histolytica* is bringing new light into our understanding of this remarkable protozoan parasite and of the disease it causes. New achievements affect the way we approach many essential questions about *E. histolytica*, from the mechanism of its pathogenicity to the definition of *E. histolytica* as a separate species from the nonpathogenic *E. dispar*. To give a single example; transfection of trophozoites is now possible and a new generation of studies taking advantage of this capability of manipulation is expected in the short term. Our goal with this review is to provide an updated and simple guide to the growing information on the molecular biology of *E. histolytica*. (*Arch Med Res* 1996; 27:403)

KEY WORDS: *E. histolytica*; *E. dispar*; Gene cloning; DNA sequences; Review.

Introduction

Amebiasis by the protozoan *Entamoeba histolytica* is one of the leading causes of morbidity and mortality in the world. It has been estimated that 10% of the global population is infected, and it is the third cause of death among parasitic diseases, only after malaria and schistosomiasis (1). In Mexico, a recent sero-epidemiological survey showed that 8.3% of individuals are seropositive (2). This figure is 2 - 2.5% higher than that obtained in a similar survey two decades ago (3).

Trophozoites of *E. histolytica* attach the intestinal epithelium producing ulcerative lesions, or penetrate to the liver and other organs causing extensive damage (4,5). Adhesion to the target cell is mediated by surface lectins of *E. histolytica* (6). Cell destruction is carried out through release of lytic mediators including proteinases (7,8), lipases (6), channel-forming peptides (9-12), among others.

Many interrogants about the pathogenic potential of *E. histolytica* still remain. For example, there is a controversy on the possibility that two morphologically indistinguishable species of *Entamoeba* might coexist, one being pathogenic: *E. histolytica* and the other simply commensal: *E. dispar* (13-15). The explanation could reside in a precise definition of the molecular components responsible for parasite pathogenicity.

The ways in which molecular biology is improving our understanding of *E. histolytica* is the subject of this review. Recombinant DNA techniques have provided us with a new version of amebiasis and of its host-parasite relationship. In the initial sections we summarize the

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influence of this new vision regarding several aspects like the evolutionary position of *E. histolytica* in the tree of life, the general organization of its genome and the possible existence of two different species of trophozoites in human isolates. In the second part, concise descriptions are given for more than 30 genes characterized to date, ranging from cytoskeletal proteins to factors of virulence and vaccine candidates. The ability to transfect *E. histolytica* achieved last year opens the way to manipulate the parasite genome for a variety of studies of gene regulation and expression.

Entamoeba: The Cell

Which is the most archaic eukaryotic organism? This is a pertinent question considering that some protozoans such as *E. histolytica*, *Giardia lamblia* or *Vairimorpha necatrix* lack mitochondria and have therefore been classified as Archezoa (16,17). Phylogenetic analysis based on sequences of 16S ribosomal genes suggests that *G. lamblia* and *V. necatrix* diverged before the advent of symbiosis with protomitochondria, while *E. histolytica* separated from the eukaryotes with mitochondria after *Euglena* (18-21). On the other hand, analyses using amino acid sequences of ubiquitin and the elongation factor 1-α indicate that *E. histolytica* appeared before eukaryotic cells acquired mitochondria (22-24). Although the contest for maximum antiquity has not yet been resolved, there is little doubt that these are organisms of early origin among eukaryotes (25,26).

E. histolytica is a structurally simple eukaryote with an unsophisticated cytoplasmic organization and nuclear division without evident chromosomal condensation. The trophozoite lacks mitochondria, Golgi apparatus, well-developed endoplasmic reticulum, typical lysosomes, and organized cytoskeleton. In fact, the only typically eukaryotic organelle of ameba is the nucleus, and even this organelle is somewhat different from the nuclei of other eukaryotes. This parasite is a remarkably mobile cell capable of ingesting a variety of particles including bacteria and erythrocytes and of destroying almost any cell. It moves by pseudopodia formation without apparent microfilaments, and microtubules are only observed during nuclear division (27). Beside the pseudopodias it also forms phylopods which are phagocytic projections with vacuoles oriented to the exterior and to a caudal uroid region. The trophozoite surface is highly active with a notable membrane turnover. Antibody-capping is probably a manifestation of a parasitic mechanism to evade the host immune response (27). Its main source of energy is carbohydrates which are incorporated through specific transporters. Despite the absence of mitochondria, the trophozoite is capable of growing under aerobic conditions of up to 5% oxygen.

The Amebic Genome

The trophozoite nucleus is 3 - 5 μm in diameter; cells with more than one nucleus may be found as well as giant multinucleated cells. The mature cyst contains four nuclei, morphologically identical to the trophozoite nucleus. The nuclear membrane remains intact during nuclear division and shows hexagonal series of pores, typically and regularly distributed (28).

Peripheral chromatin is distributed along the internal border of the nucleus and contains DNA involved in RNA synthesis (29). During interface, DNA appears disperse within the nucleus; however, before nuclear division, DNA concentrates forming the central endosome (29).

In most eukaryotes chromatin is organized in nucleosomes. The nucleosome is an octameric structure constituted by pairs of histones H2A, H2B, H3 and H4 with 160 bp of DNA rolled around the protein core (30,31). The chromatin of *E. histolytica* shows a typical structure of rosary-beads with nucleosomes of 10 nm in length. However, the electrophoretic mobility of the six basic proteins differs from the histones of other eukaryotes (32). The amino acid sequence of *E. histolytica* H3 is less than 70% similar with human histones H3.3 and H3.1, which constitutes the greatest sequence divergence for any eukaryotic H3 histone (33). It has also been proposed that *E. histolytica* does not organize all of its chromatin into nucleosomes (21,32,33).

Some studies suggest that ameba contains 5 - 8 chromosomes (34,35) while others suggest it contains 4 - 5 pairs (28,36). Pulse-field electrophoresis separates nine bands ranging from 0.3 to 2 Mb (37). Transverse alternating field electrophoresis (TAFE) resolves 11 and 17 bands in the range of 0.3 to 3 Mb. Most resolved bands are lineal molecules (38) and some could correspond to extrachromosomal DNA (39,40). On the other hand, a center of microtubular organization has been described in the nucleus of *E. histolytica* which could participate in the rearrangement of chromosomes during metaphase (35).

Total DNA determinations indicate that each trophozoite contains 0.4 - 1.5 pg (32,41-43). Genome size of *E. histolytica* is approximately 4 x 10^8 bp, with approximately 38,400 transcriptionally active genes which is eight times bigger than the number of active genes in *Drosophila melanogaster* (32). The estimated level of ploidy is 14n or more for *E. histolytica* strain HK9, 9n for strain Laredo and 1n for *Entamoeba moshkovskii* (44).

Ribosomal genes reside in more than 200 circular extrachromosomal molecules of 25 kb in length (40). Each of these plasmids is constituted by two transcriptional units of rRNA in inverted position. Each transcriptional unit measures approximately 5.9 kb and includes genes 16s, 5.8s and 25s following the typical

eukaryotic arrangement (39,45,46). In addition to the two rRNA transcriptional units, the circles code for a transcript of 0.7 kb (45). Most repetitive sequences described for *E. histolytica* have been identified within the rRNA episome (40,47-50). Nonetheless, a novel repetitive sequence has been described with units repeated in tandem located at a different part of the genome (51,52). Recently, the location of genes coding for hemolysins were described on a 2.6 kb segment of the extrachromosomal circles (53).

Entamoeba *histolytica*: One or Two Species?

There is an ongoing discussion around the question of whether pathogenic is a separate species of nonpathogenic amebas. The controversy was initiated in 1925 by Brumpt, who considered one parasitic and one commensal species of *Entamoeba* (13). Only in 1978, some criteria for differentiation of pathogenic and nonpathogenic trophozoites were based on isoenzyme patterns (54,55). The advent of recombinant DNA techniques has brought a new insight by allowing to look for differences within gene sequences. The existence of two species is supported by differences in specific sequences (56), in riboprint analysis (57,58), in clones obtained from cDNA libraries (59,60) and in ribosomal gene sequences (57,61-63). Differences in sequences, transcription levels and copy number have also been reported among pathogenic and nonpathogenic isolates, for some genes described below: actin, surface antigens, cystein proteinases, superoxide dismutase, a serine/threonine kinase and amebapore.

Some groups have described interconversion from nonpathogenic to pathogenic under certain culture conditions of trophozoites (64-68). Authors in favor of the two species argue that nonpathogenic isolates are formed by heterogeneous populations with small numbers of pathogenic amebas. It has been estimated that ten pathogenic trophozoites are enough to become established in a nonpathogenic culture under axenic conditions (69). As *Entamoeba* is without question a remarkably flexible organism, the central issue of this controversy is the distinction of whether pathogenic and nonpathogenic trophozoites are the two extremes within a continuum or two genetically isolated groups of organisms. Solution of the controversy is beyond the scope of this review and terms such as *E. histolytica* are used for the disease-causing trophozoites, whereas pathogenic or nonpathogenic will be used to apply on trophozoites or isolates only.

Cytoskeleton Proteins

Coding sequences of actin genes were the first to be isolated and characterized, this protein being the most abundant in the trophozoite (70,71). Actin had been previously purified and characterized (72,73). It shows 89% identity with the human cytoplasmic and 86% with the skeletal muscle isoforms. Actin of ameba possesses a glycine in the amino terminal only observed in two related protozoans: *Dictyostelium discoideum* (74) and *Acanthamoeba castellani* (75).

Genes coding for the heavy chain of *E. histolytica* myosin have also been identified (76,77). The protein deduced from the nucleotide sequence is 2139 bp and shows low identity with myosin from other protozoans such as *A. castellani* (39.4%), *D. discoideum* (38.4%) and even lower with myosin II from chicken smooth muscle (30%). Myosin is located at the uroidal region being involved in the movement of trophozoites and in the capping of extraneous molecules (78).

Genomic and cDNA clones of tubulin have also been obtained containing an open reading frame coding for a protein of 455 amino acids (79). *E. histolytica* tubulin lacks a polyacidic complex at the carboxy-terminal necessary for polymerization. This deficiency might explain, at least in part, the scarcity of microtubules observed in the cytoplasm of trophozoites. Some analyses have revealed the presence of two contiguous copies of tubulin in the amebic genome (79).

Surface Molecules

A cysteine-rich protein of 29 kDa, which forms dimers and oligomers through disulfide bonds, has been localized on the surface of trophozoites (80,81). A partial coding sequence has been isolated from a cDNA library using the fraction of an anti-ameba hyperimmune serum, preadsorbed to select surface antigens (80). Its predicted amino acid analysis shows homology with the precursor of a surface antigen of *P. falciparum* merozoites. Monoclonal antibodies raised against this recombinant 25 kDa fragment confirms its presence on the trophozoite surface and allows differentiation of pathogenic and nonpathogenic isolates by immunoblot (82).

Another immunogenic protein of 125 kDa also present on the surface of pathogenic and nonpathogenic trophozoites was isolated using pooled sera from patients with liver amebiasis (83). The complete coding sequence was obtained from genomic and cDNA clones. The amino acid sequence revealed a protein rich in tyrosine and asparagine with a hydrophobic region of 35 amino acids at the amino terminal which could be an anchor sequence or a signal peptide. The 125 kDa protein is polymorphic and shows variability of up to 13% between pathogenic and nonpathogenic trophozoites, and 1% between isolates of the HM1-IMSS strain.

Amebas have a mechanism of resistance against drugs and other toxic substances which might be related to surface transporters. Two genes of P-glycoproteins (mdr) have been identified in *E. histolytica*, isolated from a cDNA library prepared from a strain resistant to emetine

(84). It has been proposed that the resistance to emetine of the strain is due to over-expression of these genes. Another protein of the ABC transporter superfamily has also been identified in amebas; however, its expression is not associated with emetine resistance in *E. histolytica* (85).

A specific 60 kDa surface antigen of nonpathogenic *E. histolytica* trophozoites was cloned from an expression library through screening with monoclonal antibodies (86). This protein seems to be present only on the surface of nonpathogenic trophozoites and cysts. It is also absent in other species of amebas like *E. moshkovskii* and *E. invadens*. The possibility of using this antigen as a diagnostic tool is open. Other proteins also identified on the surface of trophozoites are SREHP, and the most studied protein of *E. histolytica*: a lectin with affinity to galactose (see below).

Proteins with Repetitive Sequences

The serine-rich protein (SREHP) is apparently specific from pathogenic trophozoites (HM1-IMSS) of *E. histolytica*, showing repetitive sequences similar to the circumsporozoite protein of plasmodium (87). Its 800 bp transcript has two overlapped open reading frames: one codes the 233 amino acids of SREHP, formed by five repeated sequences of 12 residues intercalated with four repetitive units of eight residues. Recombinant SREHP is recognized by sera from patients with amebic liver abscess or by hyperimmune sera from rabbit. It has been suggested that SREHP is involved in the adherence of trophozoites to mammalian cells. Recombinant SREHP has also been expressed in a *Salmonella typhimurium* attenuated vaccine strain capable of infecting gerbils (88).

Another example of a protein with repetitive sequences is Eh20RP, accidentally cloned during the isolation of aldehyde dehydrogenase cDNA (89). Six clones not related to aldehyde dehydrogenase had the same open reading frame coding for a 20 kDa protein, containing four almost identical repetitive units of 37 residues each. Eh20RP is abundant in the trophozoites of pathogenic and nonpathogenic amebas and appears to be not homologous with other proteins of the Database.

A family of closely related transcripts with differences within an internal region, coding for repetitive elements of 8 or 12 amino acids each, has also been isolated from pathogenic and nonpathogenic trophozoites (90). Differences in their genomic organization might be exploited to differentiate among pathogenic and nonpathogenic amebas.

Molecules Associated with Virulence

Several molecules associated with the virulence of *E. histolytica* trophozoites have been characterized, including a galactose-binding lectin, proteinases and a pore-forming protein.

Galactose-binding lectin. Adherence to the target cell is essential for the lytic action of ameba trophozoites. The surface galactose-binding lectin appears to be an essential mediator for adherence to different lines of epithelial cells including human and rat colon mucosa (91-93). This lectin is a disulfide-linked heterodimeric glycoprotein, with subunits of 170 and 35 kDa (94). The coding sequence of the heavier subunit was cloned and characterized independently by two research groups (95,96). Both clones (hgl1 and hgl2) predicted a protein of 143 kDa, but showed significant differences in their amino acid sequences (88% similarity). A third related gene (hgl3) was identified on genomic DNA by hybridization with a probe from hgl1 (97). These results suggest the existence of a family of adhesin genes in *E. histolytica* (97,98).

Sequence analysis of the 170 kDa subunit shows three well-defined regions: an external moiety of 1294-1209 amino acids with three domains: a cysteine-rich domain, a domain with nine pseudo-repetitions of 30 residues and a cysteine-poor domain. The transmembrane and cytoplasmic regions have 26 - 28 and 38 - 41 residues, respectively. This lectin subunit also shows a fragment with homology to the C8 and C9 complement components as well as to CD59, an inhibitor of complement membrane attack complex (MAC). This homology has been involved with a protective role of trophozoites against MAC (99).

The 170 kDa subunit is highly immunodominant and epitopes have been located within the cysteine-rich domain using pooled immune human sera or monoclonal antibodies (100,101). Immunization of gerbils with this subunit induces more than 65% protection against intrahepatic challenge with trophozoites. Protection appears to be mediated by both humoral and cellular responses (102). Interestingly, immunization with a fusion protein containing the cysteine-rich domain of the heavy subunit results in higher levels of protection against intrahepatic challenge with trophozoites (103). These results appoint the 170 kDa lectin subunit as a major candidate for vaccination against amebiasis (104).

In contrast, the small subunit of the galactose-binding lectin is a poorly immunogenic protein and provides the dimer with its anchoring to the membrane through phosphatidylinositol (105). Its coding sequence cDNA has also been cloned and characterized showing a cytoplasmic domain and a hydrophobic amino-terminal region that might function as a signal peptide (106). Some data suggest the existence of two populations of the small subunit which might result in two types of dimers: 170/35 and 170/30 (107).

Amebapore. The pore-forming peptide was described more than a decade ago (108,109). It is a small peptide of 8.2 kDa (77 amino acids) capable of making pores in artificial lipid bilayers (9-11). Amebapore peptides form

ionic channels in membranes by irreversible association in oligomers, similar to the C9 component of the complement cascade and to the perforin of cytotoxic T lymphocytes. Structural analysis suggests formation of four α-helices linked by disulfide bond as long as necessary to cross the bilayers of the membranes (9). Amebapore appears to be one of the major factors mediating lysis of target cells by pathogenic amebas. A 60% lower activity of amebapore has been reported for nonpathogenic as compared with pathogenic amebas (12). Sequence of amebapore from nonpathogenic amebas also shows a shorter region of hydrophobic residues and four changes in the sequence when compared to the pathogenic amebas. One of the changes (glu/pro in position 2) modifies the α-helix of the nonpathogenic amebapore and could be the cause of its reduced activity.

Proteinases. High virulence of *E. histolytica* isolates correlates with higher content of cysteine or cathepsin-like proteinases in secretion products or trophozoite extracts (110-112). Northern blot studies suggest that expression levels of one proteinase can be 10 to 100-fold higher in the pathogenic trophozoites (8). Three major groups of cysteine proteineases with similar substrate specificity and optimal pH appear to occur in *E. histolytica*. The 56 kDa, which is the main secreted proteinase, has a C3 convertase-like activity (113). The 27 kDa and 26 kDa (histolysin) proteinases are 85% similar in sequence (7,8). Variants of these proteinases (cEh-Cpp and ACP3 with MW of 27 kDa and cEh-CPnp, ACP2 and histolysin with MW of 26 kDa) have been reported in pathogenic and nonpathogenic *E. histolytica* (7,8,114,115). Another cysteine proteinase only present in pathogenic trophozoites has been described (ACP1) (114). This proteinase which is substantially different from the others (35 - 45% similarity with ACP2 and ACP3), might represent a different type of enzyme.

Metabolic and Redox Enzymes

Amebas are anaerobic fermenters degrading glucose to acetaldehyde and ethanol through glycolysis. Enzymes of the Embden-Meyerhoff pathway are targets for drugs used in the treatment of invasive amebiasis (116). Interest in the characterization of these metabolic enzymes is growing. The coding sequences for some of the glycolytic enzymes have been characterized: NADPH-dependent alcohol dehydrogenase (117,118), aldehyde dehydrogenase (118), pyruvate-orthophosphate dicinase (119), and a 95 kDa protein homologous to the adhE of *Escherichia coli* with activity of NAD+-dependent aldehyde and alcohol dehydrogenase (120,121).

Strong peroxidase and superoxide dismutase activities have long been described in several species of ameba (122). These enzymes protect trophozoites against products of the respiratory burst of phagocytes during invasion of host tissues. The superoxide dismutase activity in lysates of *E. histolytica* trophozoites is sensitive to hydrogen peroxide and to sodium azide, but insensitive to cyanide, suggesting that it is an iron-containing dismutase (FeSOD) (123). Two cDNA clones from pathogenic (cEh-FeSODp) and nonpathogenic (cEh-FeSODnp) trophozoites coding for enzymes differing in a single amino acid residue were identified.

Diagnostic Probes

Recombinant DNA provide an alternative to microscopic or immunologic identification of *E. histolytica*. DNA hybridization techniques allow differentiation among *Entamoeba* species (48) as well as among pathogenic and nonpathogenic isolates (56,66,124,125). PCR detection of *E. histolytica* in stool samples has been based on repetitive sequences, surface antigens, pathogenic zymodemes and rRNA genes (60,83,126-131). *E. histolytica* can also be detected by PCR in liver abscess fluid (132).

Recombinant proteins above have been used for immunodiagnosis of amebiasis. Recombinant SREHP is recognized by 82% of sera from patients with invasive amebiasis in immunoblot testing (133); recEh-1 of the 125 kDa antigen is recognized by 73% of sera from patients with amebic liver abscess (134) and a fusion protein with the cysteine-rich domain of the 170 kDa lectin subunit (170CR/GST) reacts with 90% of sera from patients with amebic liver abscess (100).

Miscellaneous

A number of other genes of *E. histolytica* have been characterized (see Table 1). Strategies for isolation include random selection of clones from cDNA libraries, screening of libraries with hyperimmune sera or DNA probes, PCR amplification of genomic DNA using oligonucleotide primers to conserved amino acid sequences, among others. The number of characterized genes is growing rapidly and increases the availability of molecular information in a wide range of areas in relation to the understanding of amebiasis and of *E. histolytica*.

Conclusions

The molecular information on *E. histolytica* has grown quickly over the last decade, improving our understanding on aspects ranging from intermediate metabolism to virulence factors. Several antigens are being tested in protection studies; at least one recombinant peptide appears as a promising candidate for vaccination (103,104,149).

The analysis of the cloned *E. histolytica* genes begins to disclose several general features of this parasite: the majority of genes lack introns, coding regions are rich in A:T, especially at the third position of codons, coding

Table I
Miscellaneous Genes of *E. histolytica*

Name	Code name	Size	Features	Ref.
Ferredoxin		6 kDa (59 aa)	Family with at least two genes	135
Elongation factor 1-α	EF-1α	49 kDa (430 aa)		136
Calcium-binding protein	pGENCAM	15 kDa (134 aa)	Recombinant protein binds calcium	137
Heat-shock protein	Hsp70	71.5 kDa (656 aa)	Immunodominant antigen	138
Ubiquitin		8.5 kDa (76 aa)	Highly divergent ubiquitin	22
Elongation factor 2	EF-2	93 kDa (840 aa)		139
Zinc-binding protein	EHZc3	9 kDa (84 aa)	Recombinant protein binds zinc	140,141
Protein kinase p34 cdc2	Ehcdc2	34 kDa (291 aa)	Contains an intron	142
Protein-serine/ threonine kinase	Ehrac1	47 kDa (322 aa)	Single-copy gene	143,144
rho oncogene	Ehrho1	23 kDa (208 aa)		145
rap oncogenes	Ehrap1 and Ehrap2	21 kDa (184 aa, both)		146
ras oncogenes	Ehras1 and Ehras2	21 kDa (205 and 203 aa)		146
Glutamic acid-rich antigen	K-18	39 kDa (335 aa)	Contains an intron	147
Proton-transporting ATPase catalytic subunit	Ehvma1	67 kDa (607 aa)	Catalytic peptide A	148

sequences are linked to each other with interrupting stretches of no more than 1350 nucleotides, regulatory sequences (transcription start point: ATTCA or ATCA and termination: TAA/TTT) differ from the consensus sequences of eukaryotic genes. TATA box-like sequences (TATTTAAA) are commonly localized 30 nucleotides upstream of the transcription initiation site (150).

Recently, the first successful transfections of *E. histolytica* have been carried out. Reporter genes chloramphenicol acetyltransferase (151) and firefly luciferase (152) were transiently expressed under the control of noncoding upstream sequences of the actin and galactose-binding lectin. This ability to transfect *E. histolytica* opens the door to manipulation of the parasite genome either by disrupting normal gene expression or expressing heterologous genes. Although stable transfection of *E. histolytica* is desirable, even transient transfections will aid our understanding of the regulation of its gene expression, and will surely allow a more detailed analysis of genetic factors associated with its virulence.

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A New Isoform of the Serine-Rich *E. histolytica* Protein Recognized by Human Secretory IgA Antibodies from Patients with Intestinal Amebiasis[1]

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Introduction

Intestinal infection by *Entamoeba histolytica* stimulates the local production of secretory IgA antibodies that inhibit the adherence of the trophozoites to epithelial cells, avoiding their destruction (1). Thus, a potential strategy for protection against amebiasis is the oral delivery of trophozoite proteins to induce a local IgA response. An oral vaccine-candidate is the serine-rich *E. histolytica* protein (SREHP) which contains multiple octapeptide and dodecapeptide tandem repeats (2). The SREHP is predominantly recognized by seric antibodies from patients with hepatic amebiasis; rabbit antibodies to the recombinant protein inhibit the adherence of trophozoites to mammalian cells (2, 3). Moreover, immunization with SREHP protects against development of amebic liver abscess in gerbils (4). Oral administration of a vaccine strain of *Salmonella typhimurium* expressing the complete SREHP, or the dodecapeptide fused to the B subunit of the cholera toxin induce the production of secretory IgA antibodies in mice (5). However, the production of anti-SREHP secretory IgA antibodies in patients with intestinal amebiasis has not been reported.

We have isolated clones coding for a new isoform of SREHP through the screening of a cDNA library with salivary IgA from patients with intestinal amebiasis. It is conceivable that oral delivery of SREHP in humans can raise specific IgA antibodies capable of blocking the adherence and intestinal colonization by *E. histolytica*.

Materials and Methods

Saliva Samples. Saliva from patients with intestinal amebiasis was collected at the Hospital General de México. Diagnosis was carried out by stool examination and by detection of anti-*E. histolytica* IgA antibodies in saliva. The saliva samples were pooled and centrifuged at 10,000 x g for 30 min, and the supernatant was kept frozen at -20°C until used.

Isolation of cDNA Clones. A lambda ZAP II (Stratagene USA) cDNA expression library of *E. histolytica* strain HM1:IMSS, clone A, was screened with pooled saliva samples from patients with intestinal amebiasis, extensively preadsorbed with an *Escherichia coli* cell lysate to remove undesired specificities. In brief, IPTG-treated filters were incubated overnight at 37°C on the plated library and blocked with 3% BSA-TNT (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.05% Tween 20) for 1h at 37°C. The filter-replicas were incubated overnight with the saliva at 4°C and washed three times with 1% BSA-TNT, 0.1% Nonidet P-40. Finally, the replicas were incubated with a goat anti-human IgA antibody, coupled to peroxidase (diluted 1:1000 in 3% BSA-TNT) during 2h at room temperature, and developed with 4-chloro-1-naphthol as substrate. Secondary and tertiary screenings allowed isolation of positive plaques, and their phagemids were excised for subsequent analysis, using the ExAssist helper phage/SOLR system (Stratagene USA) following instructions of the manufacturer.

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Sequencing of cDNA Clones. Sequencing of inserts within positive plasmid clones was carried out by the chain-termination method with dideoxinucleotides, using the Sequenase 2.0 System. (U.S. Biochemical Corp.). BLAST searches for homology against the sequence databases were done using nucleotide and deduced amino acid sequences of cDNA clones.

Results

In order to identify antigens recognized by the secretory IgA, we screened a lambda ZAP II cDNA library of *E. histolytica*, strain HM1:IMSS, with a pool of salivas from patients with intestinal amebiasis. Several clones were isolated and characterized; two clones (ZSP6 and ZSP7) had identical inserts of 555 bp, containing two overlapping open reading frames coding for peptides of 170 (ORF1) and 166 (ORF2) amino acids (Figure 1A). BLAST search against the GenBank database showed homology with a previously reported cDNA clone of SREHP (c1), which also contains a second ORF coding for a putative glutamine-rich protein (2). Primary structure of SREHP shows consecutive hydrophobic and charged regions starting from the amino terminal end, followed by dodecapeptide and octapeptide tandem repeats. Amino acid sequence deduced from ORF1 of Zsp6/Zsp7 includes the complete repeat region, showing two additional octapeptide repeats at the carboxy terminal end, making 6 ASSTNKPE octapeptides instead of 4 in SREHP (Figure 1B). This additional sequence also enlarges the putative glutamine-rich protein encoded by ORF2.

The coding region of ZSP6 has been subcloned in frame for SREHP into the expression vector pRSET (InvitroGene). Western blot analysis using patient salivas demonstrates a strong recognition of this new isoform of SREHP which is currently being tested in protection studies using a murine model of intestinal amebiasis (6).

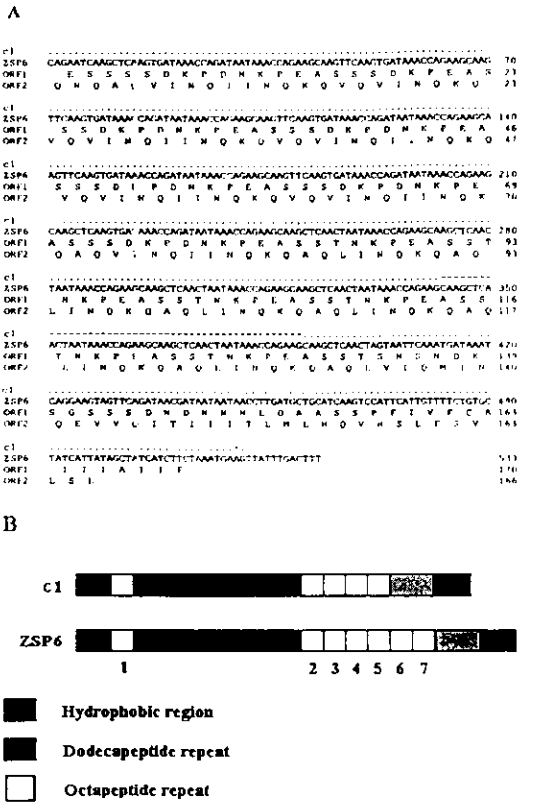

Figure 1. Characterization of a partial cDNA clone coding for a new isoform of SREHP. A) Alignment of nucleotide sequences of ZSP6 and c1 clones showing open reading frames for SREHP (Orf 1) and the putative gln-rich protein (Orf 2). Homologies are indicated by dots; dashes show the nucleotides absent. B) Schematic comparison of the repeated primary structures of SREHP deduced from ZSP6 and c1, following the style in Reference 2.

Discussion

We describe two identical cDNAclones isolated by immunoscreening of an expression library, with saliva from patients with intestinal amebiasis. To our knowledge, this is the first report of *E. histolytica* gene cloning using secretory IgA antibodies. The identification of a new isoform of SREHP adds to the list of variants of this protein (5). Recognition of this immunodominant protein by the human secretory immune response opens the possibility of testing SREHP in protection studies as an oral immunogen. Anti-SREHP IgA antibodies could inhibit the adherence of trophozoites to the colonic mucosa preventing its destruction (1,5).

Identification of other *E. histolytica* proteins recognized by the secretory response can increase our arsenal of potentially useful antigens to stimulate the mucosal immunity, as well as to understand the differences in antigen presentation to the seric and secretory immune systems occurring in amebiasis.

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KEY WORDS: *Entamoeba histolytica*; Intestinal amebiasis; IgA antibodies.

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Zinc: Interference with the Calcium Function in *Entamoeba histolytica*[1]

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Introduction

Cytolytic activity of *Entamoeba histolytica* depends on the integrity of the cytoskeleton, which is responsible for membrane movement and for expression of the receptors necessary for adherence to the target cell. Other factors, such as enzymes, lysosomal proteins and amebapore, also mediate in parasite's lytic activity. Filament movement, amebic lysosomal enzyme liberation and channel formation in the target cell require calcium (1).

Zinc acts on different elements of the cytoskeleton, besides being partially antagonic to calcium (2). Despite the importance of this effect on activities related to pathogenicity, there is no report to our knowledge on the direct effect of zinc on *E. histolytica*. A concentration of 5 mM zinc is lethal for trophozoites, while 1 mM only inhibits replication and amebic adherence to MDCK epithelial cells (manuscript in preparation). To determine whether the effect of zinc is, at least partially, due to its antagonism with calcium, we analyzed the effects of both cations on the replication and adherence of trophozoites to monolayers of MDCK epithelial cells.

Materials and Methods

Parasites. Trophozoites of *E. histolytica* strain HM1:IMSS were axenically grown in TYI-S33 medium supplemented with 15% adult bovine serum at 37°C. The trophozoites were harvested in log phase by chilling on ice for 10 min.

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Treatment of Trophozoites with EDTA. Trophozoites were incubated in TYI-S-33 medium with 10 mM EDTA for 30 min at 37°C. After the treatment, trophozoites were washed with phosphate buffer saline solution (PBS, pH 7.4) and used as culture inocula.

Cultures. 1×10^5 normal or EDTA-treated trophozoites were grown in 10 ml of culture medium either with or without: 1) 1 mM zinc (Sigma Chemical Co., St. Louis MO, USA); 2) 1 mM calcium (Baker Chemical Co); 3) 5 mM calcium; 4) 1 mM zinc and 1 mM calcium. Cultures were performed in triplicate in a total of five assays.

Viability and Proliferation. The viability tests were performed by the Trypan blue exclusion technique. Trophozoite proliferation was determined at 72 h by counting in a Neubauer chamber.

Adherence. Trophozoite adherence to MDCK cell monolayers was determined at 24 h by a previously described immunoenzymatic assay (3).

Statistic Analysis. Data are reported as the average number ± standard error. Significance was obtained by Student's t test.

Results

Amebas Treated with Zinc and Calcium

Proliferation. *E. histolytica* trophozoite viability was not affected (>90%) when either 1 mM calcium, zinc or their combination were added to the cultures. Cultures incubated for 72 h with 1 mM zinc showed significant proliferation inhibition (42 %; $p<0.001$) with respect to the untreated trophozoites. In contrast, with 1 mM calcium, the proliferation increased by 25%. Cultures treated with a combination of 1 mM zinc and 1 mM calcium

showed a slight decrease (10 %) of proliferation with respect to controls (Figure 1A).

Adherence. Treatment of cultures with 1mM zinc for 24 h caused 39 % inhibition ($p<0.001$) of trophozoite adherence to monolayers of MDCK cells. With 5 mM calcium, adherence was slightly superior to controls (15%). On the other hand, 1 mM calcium inhibited adherence by 27%. The combination of both cations (1 mM) inhibited adherence by 23% (Figure 1C).

Amebas Incubated with a Chelating Agent Previous to Zinc and Calcium

For the *in vitro* determination of the zinc and calcium effect in a cation-free system, *E. histolytica* trophozoites were treated with EDTA previous to the addition of cations.

Proliferation. Trophozoite viability was not affected during the treatment with the chelating agent (>90%); however, in 72 h cultures, proliferation diminished by 50% ($p<0.001$) related to the EDTA non-treated control. Addition of 1 mM zinc, calcium or their combination to culture, re-established the EDTA altered proliferation by 20%, 96% and 46%, respectively (Figure 1B).

Adherence. Similar to proliferation, EDTA decreased adherence of trophozoites to monolayers of MDCK cells by 44% ($p<0.001$) related to EDTA non-treated control. Addition of 1mM zinc, calcium or their combination re-established the EDTA altered adherence by 31%, 63% and 60%, respectively. Interestingly, with 5mM calcium the adherence was 30% above the non-treated controls (Figure 1D).

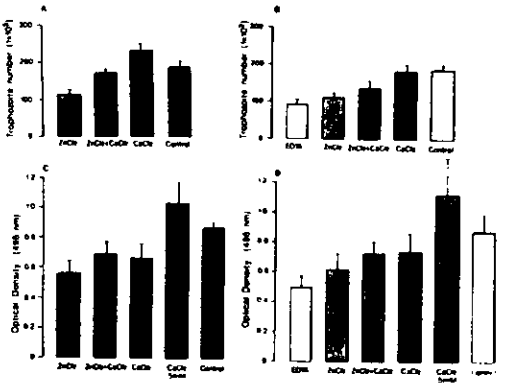

Figure 1. Effect of zinc and calcium on the proliferation (trophozoites/ml) and adherence (optical density) of *E. histolytica* trophozoites. A. Effect of zinc and calcium on trophozoite proliferation. B. Effect of zinc and calcium on the proliferation of trophozoite treated with 10 mM EDTA for 30 min prior to cation addition. C. Effect of zinc and calcium on untreated trophozoite adherence to monolayers of MDCK cells. D. Effect of zinc and calcium on the adherence of trophozoites treated with 10mM EDTA. The cations were added in 1 mM concentration, except for 5 mM calcium as indicated.

Discussion

The present study demonstrates that basic activities of the *E. histolytica* pathogenicity, such as adherence and proliferation are altered by the presence of zinc and calcium in the culture medium. It has been suggested that microtubules participate in amebic proliferation, and microfilaments in adherence (1). A dose of 5 mM zinc is lethal; however, an equal dose of calcium increased adherence. Treatment of trophozoites with 1mM zinc significantly inhibited its proliferation and adherence, which suggests that zinc exerts its effect, at least in part, on the cytoskeleton, mainly on amebic actin and tubulin polymerization (manuscript in preparation), as has also been observed for some nervous tissue cells (2). With a concentration of 1mM calcium, the adherence diminished slightly, according to a previous report (4). Our results suggest that cytoskeleton components of the trophozoites are affected in different ways by calcium, concentration. When the amebic cultures were treated with EDTA, under the conditions which did not affect trophozoite viability, before adding zinc and calcium the results showed that the cation equilibrium necessary for the correct assembly of cytoskeleton components was altered by the chelating agent. If the cations are totally or partially absent from the culture medium, addition of calcium or other cations may at least partially restore the proliferation and adherence. However, when zinc was added, recovery of the function altered by the chelating agent was not significant, possibly because the deleterious effect of the metal on the trophozoite was only reduced under these conditions. Antagonism between zinc and calcium was manifest in trophozoite proliferation; adherence results were not positive in this aspect; however, no additive effect was found between cations. Interference between these cations could be explained because amebas presumably use the calcium in the culture medium to compensate for the negative effect of zinc on the cytoskeleton. On the other hand, zinc is known to bind to some calcium transporter proteins which sometimes show more affinity to zinc and bind it with greater stability than with calcium (5).

In conclusion, zinc can affect *E. histolytica* proliferation and adherence, at least in part, through the alteration of calcium function in the cytoskeleton. Studies are in progress to determine the precise effect of this cation, and the efficacy of zinc as an antiamebic element.

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KEY WORDS: *Entamoeba histolytica;* Zinc; Calcium function.

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A 148-kDa Secretory Proteinase from *Entamoeba histolytica*[1]

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Introduction

Invasive amebiasis is the third cause of mortality worldwide caused by parasitic diseases. Mechanisms that allow tissue penetration are not well understood; secreted proteolytic enzymes, a galactose-specific adhesin, extracellular matrix receptors and cytotoxic factors have been proposed to play a significant role in tissue invasion. Such factors cause lysis of epithelial cells and intestinal mucosa ulceration (1).

The release of histolytic cysteine proteinase from *Entamoeba histolytica* correlates with pathogenicity of axenic strains, it is not involved in the cytotoxic effect but is essential for the cytolytic effect (1). Secreted proteinases seem to play a key role in invasion; however, their nature, their number or their role in damage and degeneration of cells and dissolution of the basement membrane are not well known. Due to the role played in amebic pathogenicity, proteases appear to be promising targets for the development of new antiparasitic chemotherapy. In this work we searched for secreted proteinases from the highly virulent strain of *E. histolytica* HM1-IMSS.

Materials and Methods

***E. histolytica* Cultures.** Axenic cultures of *E. histolytica*, HM1-IMSS strain, were grown in TYI-S-33 medium supplemented with 15% bovine serum, 100 U of penicillin per ml-100 µg of streptomycin sulfate per ml-0.25 µg of amphotericin B per ml at 37°C in culture flasks.

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Proteinase Assays. To obtain secreted proteins, trophozoites were harvested by chilling the flasks on ice after 48 h of growth and centrifuging 10 min at 150 x g; cell pellets were resuspended and washed three times with phosphate buffer (PBS-pH 7.2) in order to eliminate culture medium to avoid proteolytic activity associated with serum components. Cell pellets were resuspended again and trophozoites were mantained in culture medium without bovine serum or vitamin mixture at 37°C; cultures were centrifuged for 10 min at 150 x g and samples were obtained from the supernatant within intervals of 1 to 6 h and kept at -20° until use. Viability was judged by Trypan blue exclusion.

Determination of Proteolytic Activity. Proteolytic activity was determined as described by Heussen and Dowdle. In brief, supernatants were electrophoresed in 0.1%-gelatin/10%-PAGE-SDS at 4°C. After staining with Coomassie brilliant blue, clear bands or zones could be seen in the gels where active gelatinases were located. No reducing agent was used in order to determine activity of native secreted proteinases. Molecular weights of *E. histolytica* proteinases were determined by their electrophoretic mobility.

Results and Discussion

Proteolytic activity reached its maximum after 2 h of incubation and decreased at 3 and 4 h, stabilizing at 6 h. These data correlate well with the damage of the cecal mucosa produced after 110 to 160 min of exposure to amebic lysates, previously reported (2).

Amebas maintained >90% viability as judged by Trypan blue exclusion. Three intense bands of 75, 56 and 42-kDa and three faint bands of 148, 30 and 28-kDa were detected. The major proteolytic activity was concentrated between 75 and 42 kDa (Figure 1). The 56-kDa protein represents the main proteinase secreted by tro-

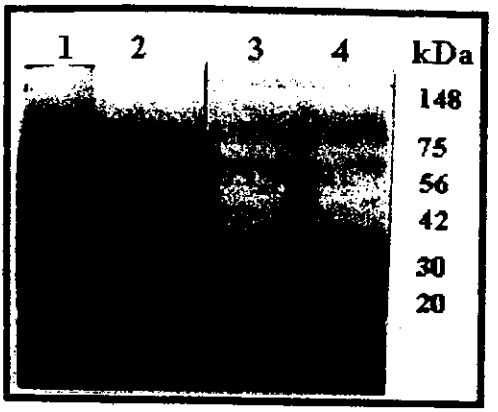

Figure 1. SDS-gelatin-PAGE of ameba secretory proteinases. Samples from the supernatant at 2 h of incubation were electrophoresed (Lanes 3 and 4) and digestion sites were detected after staining with Coomassie-blue. Lane 2 shows there was no intrinsic proteolytic activity in the culture medium without serum.

phozoites of *E. histolytica*, which has been previously described as a thiol enzyme with highest activity at neutral pH (1).

Enzymatic proteolysis has been thoroughly studied in this parasite and correlated with trophozoite virulence: function of the 56-kDa cystein-proteinase appears to be essential for damage to the intestinal mucosa (2) and consequently for the invasive capacity of the parasite and amebic liver abscess formation (3). It has also been shown that high-virulence strains possess greater proteolytic activity (4).

Bands of varying molecular sizes ranging between 76 and 25 kDa have been reported (4). We found a secretory proteinase of 148-kDa not reported before, possibly because it is rapidly degraded in the supernatant mixture or because its proteolytic activity decreases in 5 to 25% each day when stored at 4°, -20° or -70°C. It has been suggested that the smaller bands are the result of digestion of a larger protease; but no such protein of high molecular weight has been reported. Our results support this hypothesis; however, it should be kept in mind that it could be just protease aggregation resulting in apparent higher molecular weight. Interestingly, Avila et al. (5) found a surface-protein from *E. histolytica* of 150 kDa with proteolytic activity, which could correspond to the same protein we are reporting; if so, this protein could be released from the trophozoite membrane to the extracellular medium having a more complex role in host tissue destruction and in parasite virulence. Minor differences in the size of the protease could be due to the different experimental conditions.

In conclusion, proteinases are involved in the pathogenesis of invasive amebiasis and in tissue invasion mainly because of their capacity to digest a wide range of substrates including extracellular matrix components. Efforts are under way to elucidate the nature of *E. histolytica* proteinases and the mechanisms which determine their destructive potential. Here, we report an amebic secretory proteinase of high molecular weight that could also be related to the membrane and play an important role in invasion.

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KEY WORDS: *Entamoeba histolytica*; Secretory proteinase; Proteases.

ENVIADOS A PUBLICACIÓN

1. Carrero JC, Petrossian P, Acosta-Camarena E, Sánchez-Zerpa M, Laclette JP, Ortíz-Ortíz L. 1997. Cloning and characterization of *Entamoeba histolytica* antigens recognized by humna secretory IgA antibodies; preliminary protection assays with recombinant antigens against intestinal infection in mice. Enviado a Infection and Immunity.

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Cloning and characterization of *Entamoeba histolytica* antigens recognized by human secretory IgA antibodies; Preliminary protection assays with recombinant antigens against intestinal infection in mice.

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Abstract

Intestinal infection by *Entamoeba histolytca* induces a local response with production of specific IgA antibodies which can block adherence of trophozoites to mammalian cells. We have identified several antigens of *E. histolytica* recognized by secretory IgA antibodies, through an immunoscreening of a cDNA expression library, with saliva from patients with intestinal and hepatic amebiasis and with saliva from an asymptomatic cyst carrier. The antigens identified include two well known membrane proteins and vaccine candidates: the serine-rich and the 29 kDa cysteine-rich proteins, as well as five intracellular and three newly described proteins of *E. histolytica*: cyclophilin, the ribosomal L23a protein and a glutamic acid-rich antigen (EHSALJC1) which has no homologues in the protein databases. Northern blot analysis revealed single transcripts for cyclophilin and the ribosomal L23a protein, and at least four transcripts for EHSALJC1. An initial protection study against intestinal amebiasis on C3H/HeJ mice was carried out by oral immunization with recombinant fragments of the 29 kDa cysteine-rich protein and the EHSALJC1. A good correlation between the development of secretory and systemic anti-*E. histolytica* responses with the level of protection was observed. These results suggest that the trophozoite antigens which induce secretory responses in humans, can be used in the development of an oral vaccine against amebiasis.

Introduction

Intestinal amebiasis by the protozoan *Entamoeba histolytica* is one of the main causes of death by parasites worldwide. It is estimated that about 10 % of the world population is infected resulting in more than 40,000 deaths per year (52). Fortunately, a series of observations suggest that vaccine development against amebiasis is achievable (46). Intestinal amebiasis stimulates mucosal production of secretory IgA antibodies (sIgA), which can be detected in feces(17), calostrum(5), milk(22) and saliva(16). Salivary IgA antibodies inhibit the adherence of the trophozoites to epithelial cells preventing their cytolytic activity (7).

Secretory IgA responses induced by mucosal stimulation have been shown to be protective against viral, bacterial and parasitic infections e.g. caused by *Salmonella typhimurium* (33) *Vibrio cholerae*, *Campylobacter coli*, *C. jejuni*, *S. mutants* (10), *Toxoplasma gondii* (32) and *Trichinella spiralis* (15), among others. Oral delivery of *E. histolytica* trophozoite proteins that induce local IgA responses could also be protective against intestinal amebiasis. Besides, mucosal immune responses can trigger concomitant systemic responses in humans, increasing the feasibility of developing a successful vaccine (23,25,53). Induction of sIgA responses to the *E. histolytica* serine-rich protein (SREHP) and to the 170 kDa galactose-binding lectin by oral immunization of rodents has been reported (27,46,55). The sIgA against both proteins block the *in vitro* adherence of trophozoites to mammalian cells. However, with the exception of the galactose-binding lectin, the identity of antigens recognized by human sIgA remains unknown (26).

We describe here the identification of eight trophozoite antigens recognized by human sIgA. Several clones were isolated by screening of an expression cDNA library of

E. histolytica strain HM1:IMSS, using salivary IgA antibodies from patients with intestinal or liver abscess infections by *E. histolytica*, as well as from asymptomatic cyst carriers. Amino acid sequences deduced from characterized cDNA clones allowed the identification of previously reported *E. histolytica* proteins: SREHP (47), 29 kDa cysteine-rich protein (49), 70 kDa heat-shock protein (35), cysteine proteinase (41), enolase (3) and a new isoform of the acetaldehyde/alcohol dehydrogenase (6). Three novel *E. histolytica* proteins were also isolated and characterized: the cyclophilin (cyclosporin-binding protein), the ribosomal protein L23a, and a glutamic acid-rich protein (EHSALJC1) with no homology with any sequence in the protein database.

Recombinant cysteine-rich and the EHSALJC1 peptides were expressed in bacteria, purified by metal-binding affinity chromatography, and reacted in Western blot against pooled saliva from patients and from an asymptomatic carrier. The cysteine-rich protein was only recognized by antibodies from patients with intestinal amebiasis and liver abscess, whereas the EHSALJC1 was recognized by the saliva from the patient with liver abscess and from the asymptomatic carrier. Results from initial protection studies against intestinal amebiasis in a murine model, through oral immunization with recombinant 29 kDa cysteine-rich protein and EHSALJC1, suggest that an anti-ameba vaccine is feasible. Identification of parasite antigens that stimulate human mucosal responses and characterization of differential recognition among patients and asymptomatic carriers, could provide useful information for the development of an oral-vaccine and new diagnostic tools for amebiasis.

Materials and Methods

Saliva samples. Saliva samples from patients with intestinal amebiasis and from one patient with amebic liver abscess were collected at the Hospital General de México. Diagnosis of intestinal amebiasis on suspect patients was carried out by coproparasitoscopic analyses, rectosigmoidoscopy detecting the characteristic ulcers in the colonic mucosa, and through detection of anti-*E. histolytica* antibodies in saliva by ELISA (16). Diagnosis of amebic liver abscess was carried out by ultrasound imaging, corroborated by microscopic observation of trophozoites in hepatic biopsy samples. Saliva from an asymptomatic cyst carrier containing detectable anti-*E. histolytica* IgA antibodies was also obtained. The saliva sample from the patient with amebic liver abscess and from the asymptomatic carrier as well as the pool of the saliva samples from patients with intestinal amebiasis were centrifuged at 10,000 x g for 30 min and the supernatant was kept frozen at -20°C until used.

Screening of the cDNA library. The *E. histolytica* cDNA library established in λZAPII (Stratagene, USA) was used to isolate the cDNA clones recognizable by human sIgA. IPTG-induced plaques produced on *Escherichia coli* XL1-Blue were immunoscreened on separate replicas with the (i) asymptomatic cyst carrier, (ii) intestinal amebiasis, and (iii) liver abscess patients saliva samples pre-absorbed with an *E. coli* lysate to remove undesired specificities, as previously described (8). Positive plaques were isolated after three more rounds of rescreenings, and the recombinant pBlueScript SK phagemides were excised from the λZAPII positive clones using the ExAssist helper phage (Stratagene, USA) in accordance to the instructions of the manufacturer.

DNA sequencing. Sequencing of the excised phagemides was carried out by the dideoxy chain termination method (43) using the Sequenase 2.0 System (United States Biochemical Corporation) and [α-^{35}S]dATP (Amersham International plc). All sequence data were obtained for both strands of DNA. Computer analysis of the sequences was carried out with the aid of the PC/Gene program from Intelligenetics, Inc. The nucleotide and deduced amino acid sequences of the cDNA clones were analyzed for homologies using the BLAST service of the NCBI database.

Construction of pRSET-Zsaljc1 and pRSET-Zsp1. The complete cDNA inserts of immunoscreening isolated Zsaljc1 and Zsp1clones coding for EHSALJC1 and the 29 kDa cysteine-rich protein, respectively, were released by BamHI + XhoI digestion from their phagemids and the fragments purified from 1% agarose gel after electrophoresis. The fragments were ligated to the BamHI-XhoI sites of pRSET expression vector (InVitrogen Corp., USA) in frame with the ATG of the β-galactosidase gene stretch to produce fusions with the 6 x His tag of the vector. The resulting constructs pRSET-Zsaljc1 and pRSET-Zsp1 used to transform XL1-Blue cells by electroporation. The transformant colonies were selected by growth in the presence of 100 μg per ml ampicillin. The in-frame ligation of the cDNA inserts was confirmed by sequencing of the recombinant plasmids using the TCATCATGGTATGGCTAGC oligonucleotide designed to anneal to the 5' flanking region of the pRSET polylinker. The expression constructs additionally contained the BamHI-EcoRI stretch of pBluesript SK phagemid polylinker and the adaptor GGCACGAG preceding the cloned cDNA sequences. Because Zsaljc1 ORF lacks stop codon, the

expressed recombinant EHSALJC1 will contain 35 additional C-terminal residues encoded by the vector.

Expression and purification of E. histolytica antigens. To express the fusion EHSALJC1 and 29 kDA cysteine-rich peptides the InVitrogen Xpress System Protein Expression protocol was followed. The overnight cultures of the recombinant *E. coli* XL1-Blue(pRSET-Zsaljc1) and XL1-Blue(pRSET-Zspl) grown in the presence of 100 μg/ml ampicillin and 20 μg/ml tetracyclin were diluted 1:50 into SOBM broth containing 100 μg/ml ampicillin and grown for 2 h at 37 °C with vigorous shaking (OD_{600} = 0.3). The cells were induced to express fusion peptides by addition of M13-T7 helper-phage in the presence of 2 mM IPTG. The fusion proteins containing poly-hystidine tag were purified by metal-binding affinity chromatography using Hi Trap Chelating columns (Pharmacia Biotech.) treated with nickel chloride. After washings, the bound peptides were eluted with EDTA and the purity of the fusion products was assayed by SDS/PAGE (29). Protein concentration were determined by the Lowry method using bovine serum albumin as standard (31).

SDS-PAGE and Immunoblotting. Purified EHSALJC1 and 29 kDa cysteine-rich fusion proteins were separated on 12% SDS/PAGE slabs and transferred to nitrocellulose membrane (50). Nitrocellulose strips were blocked with 3% BSA in PBS for 1 h at 37°C, and incubated overnight at 4°C with the pooled saliva from patients with intestinal amebiasis, with saliva from the asymptomatic carrier or with saliva from the patient with amebic liver abscess. The strips were washed three times with 1% BSA, 0.3% Tween in

PBS and incubated with a peroxidase conjugated goat anti-human IgA antibody (1:1,000 dilution; Zymed Laboratories, Inc. San Francisco, Cal.) for 2 h at rt. After washing as above, the strips were revealed with 4-chloro-1-naphtol.

Northern blot analyses. Poly-A mRNA was purified from 10^8 throphozoites of *E. histolytica* strain HM1:IMSS by guanidinium isothiocyanate extraction and chromatography through oligo(dT)-cellulose using the QuickPrep Micro mRNA purification kit, (Pharmacia Biotech, USA). Poly-A mRNA (5 μg per lane) was electrophoresed in 1.2 % agarose-formaldehyde gels, and blotted to Hybond-N membranes (Amersham International plc). The blots were probed with agarose-purified cDNA inserts, radiolabeled with [^{32}P] dCTP using RTS RadPrime DNA labeling system (Life Technologies, USA). Final washing conditions were 0.5 x SSC/ 0.1% SDS at 60 °C.

Mice. Male inbred, 8 weeks old, C3H/HeJ mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in sterile plastic cages under air filtration with food and water *ad libitum*. All mice were parasite (especially *Entamoeba muris*) free in serial coproparasitoscopic analyses.

Immunization of mice with the recombinant antigens. Male C3H/HeJ mice were orally immunized by intragastric intubation under light ether anesthesia on days 0, 7 and 21. Each dose contained 250 μg of 29 kDa cysteine-rich or EHSALJC1 fusion peptides and 10 μg of cholera toxin (CT) (Sigma) in 0.25 ml of 0.2 M $NaHCO_3$, pH 8.3. One intraperitoneal boost with 25 μg of the fusion peptides emulsified with incomplete Freund's adjuvant (Gibco

Laboratories) was done on day 14. Control mice received bicarbonate buffer with CT and PBS with incomplete Freund's adjuvant. Fecal samples collected 25 days after immunization were tested for anti-*E. histolytica* IgA antibodies by ELISA (14). Microplate wells were coated with 10 μg of total trophozoites extract and anti-mice IgA antibody conjugated to horseradish peroxidase was used as second antibody (diluted 1:1000; Zymed Laboratories, Inc.). Each fecal sample was tested in duplicate.

Intracecal challenge with E. histolytica trophozoites. Control and immunized mice were challenged intracecally after 28 days of immunization with 5×10^5 trophozoites recovered after three passages from liver abscess in hamsters (21). In brief, mice were anesthetized with Ketamine (90 mg/kg) and Xilazyne (10 mg/kg) and the abdominal area was shaved. A laparotomy was done under aseptic conditions to expose the cecum. Trophozoites resuspended in 0.1 ml PBS were directly injected into cecum toward its apex. The orifice produced by the injection was blocked with cotton and the cecum was carefully returned to the abdominal cavity. The abdominal wall was closed with surgical suture (Vyclil 4-0) and the skin, with clips. Mice were sacrificed on day 20 post-inoculation which is the time when larger lesions are observed (21). Fecal samples and sera from mice were collected to assay for anti-*E. histolytica* antibodies by ELISA, as described above. The ceca were removed, fixed in 10% buffered formalin, pH 7.2, and embedded in paraffin; tissue sections were stained with hematoxylin-eosin for examination and photography under a light microscope.

The EHSALJC1 sequence included in this paper has been submitted to GeneBank with accession number U66671.

Results

In order to identify the antigens recognized by the human sIgA antibodies, a cDNA library of *E. histolytica* was immunoscreened with a pool of saliva from patients with intestinal amebiasis, amebic liver abscess or saliva from an asymptomatic carrier. A total of ten positive clones were isolated and characterized (Table I).

Clones isolated with saliva from patients with intestinal amebiasis. Four clones were isolated and characterized. Two clones (Zsp6 and Zsp7) had identical inserts of 555 bp, containing two overlapping open reading frames (ORF) coding for peptides of 170 and 166 amino acids. Blast-p searches with this 170 amino acid sequence showed homology with the serine-rich *E. histolytica* protein [SREHP; (47)], except for 16 additional amino acid residues in the region of repetitive sequences in tandem. The SREHP was describe as a protein of 25 kDa with five dodecapeptide and four octapeptide tandem-repeats at the carboxy terminus, whereas both of our clones show two additional octapeptide repeats, making 7 dodecapeptides instead of 5 (8). Other clone (Zsp1) with an insert of 717 bp contains an ORF encoding a peptide of 227 amino acids identical to the 29 kDa cysteine-rich protein of *E. histolytica* (49) starting 7 amino acids downstream the initial methionine, with a single nucleotide change in the position 209 (A for G), resulting in a single amino acid substitution (L for R). Finally, the fourth characterized clone Zsp8, contains a 620 bp insert including an ORF encoding a peptide that corresponds to the 1-α elongation factor (13).

Clones isolated with saliva from one patient with amebic liver abscess. Six clones were characterized. The clone designed Zsaha1 shows an insert of 327 bp coding for the 108

carboxy terminal amino acids of the glucolytic pathway enolase (3). Other clone, Zsaha4, contains an insert of 941 bp including an ORF that encodes a sequence of 308 amino acids corresponding to a cysteine proteinase (41). Two more clones were found to encode peptides with homology to known *E. histolytica* proteins albeit showing significant differences: Zsaha2 has an insert of 708 bp with a single ORF encoding a sequence of 236 amino acids which is 50-60% similar to an internal peptide of the 70 kDa heat-shock protein (HSP70) of *E. histolytica* SFL-3 strain (35). Ours is the first report of a HSP70-like protein in the HM1:IMSS strain and might correspond to a new cognate of this protein family. The Zsaha3, with an insert of 592 bp, contains an ORF coding for a 191 amino acid peptide, with moderate homology (40-50% similarity) with the acetaldehyde/alcohol dehydrogenase [EhADH2; (6)]. This finding might indicate the existence of a protein family analogous to the multifunctional *adhE* gene products of *Escherichia coli* and distinct to the NAD^+ and $NADP^+$ alcohol dehydrogenases of *E. histolytica*.

Other two cDNA clones were isolated (Zsaha51 and Zsaha54), containing coding sequences for two novel proteins of *E. histolytica*. Clone Zsaha51 contains an insert of 383 bp with an ORF coding for 119 amino acids and a 21 bp long 3´non-coding region (Fig. 1A). Blast-p searches indicated that the amino acid sequence deduced from cDNA corresponds to the ribosomal L23a protein of *E. histolytica* (EhRP-L23a), showing 58% identity and 74% similarity to the human and rat homologues (Fig. 2A). The L23a protein is part of a group of proteins involved in the initiation of the assembly of the large ribosomal subunits (18). The EhRP-L23a fragment shows the sequence KKAFVRL in the carboxy terminal end (positions 97 to 103) which closely resembles the highly conserved motif (KKAYVRL) involved in the binding to the rRNA (Fig. 2A). The terminal leucine

residue is critical for the binding of the L23a protein to the domain III of the 25S rRNA (42). The other ribosomal protein described in ameba, RP-L21, was found associated to one of the actin genes in the genome (36).

Clone Zsaha54 contains a small 242 bp insert, with a single ORF coding for 75 amino acids followed by a short 3′ non-coding region of 15 bp before the poly-A tail (Fig. 1B). Blast-p searches of the deduced amino acid sequence indicated homology with the group of highly conserved eukaryotic cyclophilins (CyPs). These proteins together with the FK506-binding proteins (FKBPs), constitute the family of immunophilins, enzymes with peptidyl-prolyl *cis-trans* isomerase activity, which act as intracellular receptors for immunosuppressive drugs like cyclosporin A (CsA), FK506 and rapamycin (51). The amino acid sequence deduced from Zsaha54 corresponding to the *E. histolytica* CyP (EhCyP), shows all conserved motifs present in the carboxyl terminal end of CyPs, involved in CsA binding, including the essential tryptophan residue (Fig. 2B) (30,51). Our partial sequence of EhCyP is 59% identical to the CyPs of human, 53% to *Plasmodium falciparum*, 65% to yeast and 74% to rice, among others (Fig. 2B).

Clones isolated with saliva from an asymptomatic carrier. Two identical clones (Zsaljc1 and 2) were isolated from the cDNA library using saliva from an asymptomatic cyst-carrier. Inserts were 546 bp long containing a single ORF coding for 182 amino acids including a poly-A stretch of 21 bp in the 3′ region. This ORF was found to lack the stop codon (Fig. 1C). No similarity has been found with the sequences in the protein database. Deduced amino acid sequence of EHSALJC1 shows a high content of glutamic acid residues (14.3%). The A/T content of Zsaljc1 is 66%, in agreement with the average for *E.*

histolytica coding sequences (37). Computer analysis of the coding sequence of Zsaljc1 using the GeneWorks program (IntelliGenetics), revealed a hydrophobic region at positions 125 to 136 which could correspond to a transmembrane domain, as well as a potential N-glycosylation site on position 72.

Transcript size for EhRP-L23a, EhCyP and EHSALJC1. To determine the transcript size of the three novel proteins of *E. histolytica*, Northern blots of trophozoite poly-A RNA were probed with the complete inserts of each Zsaha51, Zsaha54, and Zsaljc1 clones. Single transcripts of 0.6 and 0.79 kb were observed for EhRP-L23a and EhCyP, indicating that coding regions within both cDNA clones are partial (data not shown). In contrast, Zsaljc1 hybridized with at least four mRNA species ranging from 0.9 to 2.1 kb (0.9-1.3, 1.5, 1.7 and 2.1 kb approximately), suggesting that EHSALJC1 could be one isoform within a protein family (data not shown). Hybridization band sizes for EhRP-L23a and EhCyP are consistent with transcript sizes described for their homologues in different species (48,40).

Recombinant expression and purification of EhSALJC1 and of 29 kDa cysteine-rich protein. The complete inserts of Zsaljc1 and Zsp1 containing the partial coding regions EhSALJC1 and 29 kDa cysteine-rich protein, respectively, were subcloned in frame into the expression vector pRSET. After transformation, XL1-Blue(pRSET-Zsaljc1) and XL1-Blue(pRSET-Zsp1) cells were induced to express the *E. histolytica* peptides as fusion products with a metal-binding poly-histidine tag. Recombinant EHSALJC1 will contain 35 additional C-terminal residues, encoded by the vector, following the poly-lysine sequence at the 3' end of insert (see Fig. 1C). The fusion peptides showed the expected sizes of 24 and

29 kDa by SDS-PAGE of induced bacterial lysates (Fig. 3A). The fusion products were purified by metal-binding affinity chromatography resulting in more than 85% purity as determined by SDS/PAGE (data not shown).

Antibody recognition of recombinant antigens. The purified recombinant fragments of EhSALJC1 and 29 kDa cysteine-rich protein were tested for IgA antibody recognition in Western blot assays with the saliva samples used for the screening of the cDNA library. In correlation with the saliva sample that allowed isolation of the respective cDNA clone, only the 29 kDa cysteine-rich protein was recognized by the saliva from patients with intestinal amebiasis and only EHSALJC1 was recognized by the saliva from the asymptomatic cyst-carrier (Fig. 3B). However, both recombinant peptides were recognized by the salivary IgA antibodies from the patient with amebic liver abscess (Fig. 3B). These results may reflect differences in antigen recognition by the mucosal immune system of patients and asymptomatic cyst carriers.

Protection studies. As an initial evaluation of the protective potential of an oral immunization with the recombinant fragment of the 29 kDa cysteine-rich protein and the EHSALJC1, protection studies were carried out in a murine model of intestinal amebiasis, which is highly reminiscent of its human counterpart (21). Antibodies in supernatants of feces and in the sera were assayed by ELISA with a trophozoite lysate. Oral administration of each recombinant fragment, accompanied by CT, induced strong seric and secretory responses in C3H/HeJ mice after 28 days (before challenge with trophozoites) of the initial immunization. The seric response remained high on the 48th day (time of sacrifice),

whereas the secretory one decreased considerably. In contrast, the control group receiving only CT did not develop any response against the lysate (Fig. 4). Similar results were observed in supernatants of *in vitro* cultures of Peyer's patches (not shown).

Coproparasitoscopic analyses found living trophozoites in the feces of 60% of mice in the control group even 20 days after infection, whereas no trophozoites were detected in the feces of any mice in the immunized groups. The ceca of infected mice appeared swollen and contracted and the cecal contents were mucoid and yellowish, showing motile trophozoites on microscopic examination. In contrast, the ceca of immunized mice were not different from the healthy controls and did not contain trophozoites (data not shown). The epithelium of infected mice showed large infiltrates of neutrophils and lymphocytes in the submucosa and in the lamina propia, with necrosis of the mucosa and destruction of the surface epithelium and glands (Fig. 5). Many intact trophozoites were also observed among the cellular infiltrate, in agreement with our previous report (21). The ceca of most mice immunized with the recombinant 29 kDa cysteine-rich protein were indistinguishable from the bystandard controls and did not show any trophozoites, whereas moderate cellular infiltrates without trophozoites, and little evidence of damage of the surface epithelium was observed in the EHSALJC1 immunized mice (Fig. 5).

Discussion

We have isolated and characterized cDNA clones containing partial coding regions for several *E. histolytica* antigens that are recognized by sIgA in humans. The sIgA in external secretions are the first specific defense against some pathogens and have been shown to be protective in viral, bacterial and parasitic infections (reviewed in 28). In amebiasis, the biological role of sIgA is largely unknown, however, several lines of evidence suggest their involvement in protection. For example, a positive correlation between the presence of sIgA and a low incidence of amebiasis has been observed in breast-fed children in endemic areas (20,24). In a previous report, we showed that the human sIgA block the adherence of trophozoites to mammalian cells (7). Because cell adherence is supposed to be an essential step for tissue destruction by *E. histolytica* (39), our finding points out the protective potential of sIgA; nevertheless, evidence is still inconclusive.

One possibility for vaccine development against amebiasis is the use of parasite antigens capable of inducing a local IgA response in humans. However, with the exception of the galactose-binding lectin (26), the identity of these *E. histolytica* antigens recognized by human sIgA is missing and their molecular weights remain the only feature available. In this report, ten antigens of *E. histolytica* recognized by sIgA were identified. In contrast with our previous Western blot results using saliva from patients (7), the number of antigens recognized as positive on immunoscreening of a cDNA library is remarkably low. As the saliva samples were repetitively absorbed with a bacterial lysate to avoid background during screening, it is possible that many anti-ameba IgA specificities were also removed by shared antigens (2). Our results indicate that the secretory immune response of humans is significantly directed to intracellular proteins of *E. histolytica*, because at least seven out of

ten characterized antigens resulted to be cytoplasmic proteins (cysteine proteinase, enolase, heat-shock protein, acetaldehyde/alcohol dehydrogenase, 1-α elongation factor, cyclophilin and the ribosomal protein L-23a). The recognition of intracellular components correlates well with previous findings demonstrating that the ribosomal and lysosomal components represent the most antigenic fractions of *E. histolytica* (4,45). Cytoplasmic components are also prominently recognized by sIgA from patients in immunolocalization studies (34). The recognition of inaccessible antigens by the humoral immune response can be envisioned either as resulting from the release of intracellular components from unsuccessful trophozoites, or as an evasion mechanism of the host immune attack. Nevertheless, putative cytoplasmic antigens from trophozoite lysosomal and ribosomal fractions have been shown to confer protective immunity (4,45).

Two more antigens that induce local IgA responses in humans correspond to the membrane proteins SREHP and 29 kDa cysteine-rich protein, previously described as potent immunogens (46). None of them had been shown to be recognized by human sIgA although the SREHP has been successfully tested as an oral immunogen in rodents (55). The recognition of both antigens by sIgA suggests that they can be used as human oral immunogens. Other strongly immunogenic surface-proteins like the 170 kDa galactose-binding lectin and the 125 kDa protein were not isolated in this study although several lines of evidence demonstrate the recognition of the 170 kDa galactose-binding lectin by sIgA from patients with intestinal amebiasis and liver abscess (7,26). Finally, the isoform of SREHP we describe here suggests that the idea of using a single PCR on the repetitive regions of SREHP, to differentiate between pathogenic *E. histolytica* and the non-

pathogenic *E. dispar* (9) should be revised. The presence of at least two isoforms of SREHP in the same species could obscure the interpretation of the PCR results.

Human sIgA also allowed isolation of three cDNA clones with coding regions for previously unreported proteins of *E. histolytica*: EhCyP, EhRP-L23a and EHSALJC1. Among them, the cyclophilins deserve special mention. Although the biological function of cyclophilins is not well understood, they are currently being tested in studies of anti-parasite drug-design, as CsA has been shown to be effective against *Plasmodium*, *Schistosome*, and *Toxoplasma*, among others (11). This effect may be mediated by a direct action on parasite's cyclophilin or by modulation of the host immune response (11). Preliminary results indicate that *in vitro* treatment of *E. histolytica* with low doses of CsA slows their multiplication, whereas higher doses kill the trophozoites (Carrero J.C., personal communication). Current work is directed to characterize EhCyP function in terms of its isomerase activity, CsA-binding and *in vivo* modulation of the amebic infection.

The only cDNA clone isolated with sIgA from saliva of one asymptomatic cyst carrier (EHSALJC1) codes for a putative glutamic acid-rich protein without homology to any protein in the database. We don't know if the lack of a stop codon is due to a misplaced annealing of the oligo(dt) reflecting the presence of a genuine poly-A stretch in the coding sequence of EHSALJC1, or it is due to artifacts during cDNA synthesis and cloning. However, the recognition of recombinant EHSALJC1 peptide by the same IgA saliva antibodies that allowed isolation of the initial Zsaljc1 clone indicates that the relevant epitopes are located in the fragment encoded by the cDNA rather than in the C terminal amino acid sequences encoded by the vectors.

Detection of at least four transcript sizes in the Northern blot analyses, using the complete insert of EHSALJC1 as probe, suggests that a family of EHSALJC1-related proteins exists in *E. histolytica*. Another glutamic acid-rich antigen of 39 kDa (K18) was isolated from an cDNA expression library of *E. histolytica* strain SFL-3 (38). Although both proteins are glutamic acid-rich and antigenic, EHSALJC1 and K18 do not seem to be related by comparative sequence analysis.

There was no cross-isolation of clones during the screening of the cDNA expression library with the three saliva samples included in this study. Moreover, there was no cross-recognition among recombinant 29 kDa cysteine-rich and EHSALJC1 peptides in the Western blot analysis with saliva from the patients with intestinal amebiasis and from the asymptomatic cysts carrier. Thus, sIgA from patients with enteric infection and asymptomatic carriers appear to recognize different parasite antigens. In contrast, the saliva from a patient with hepatic infection recognizes both recombinant peptides. Zymodem patterns used to differentiate *E. histolytica* from *E. dispar* allowed to demonstrate that asymptomatic individuals can be carriers of *E. histolytica* cysts (19). The asymptomatic cyst-carrier included in the present study, was not characterized in terms of the species of *Entamoeba* present in the feces, however, the lack of recognition of EHSALJC1 by saliva from patients, points to differences in the pattern of antigen recognition among patients and the asymptomatic cyst carrier rather than to the carrier being infected with *E. dispar*. Therefore, a differential recognition of antigens might well be associated with resistance or susceptibility to infection by *E. histolytica*.

Susceptibility to invasive amebiasis can be associated with the genetic background of the host (1). Asymptomatic carriers of *E. histolytica* could recognize a set of antigens

that induces a protective response, whereas patients recognize another set leading to an irrelevant response. Characterization of differences in sIgA antigen-recognition among patients and asymptomatic carriers could prove useful for the identification of candidate-antigens for oral-vaccine.

An initial protection assay against the intestinal infection was carried out using susceptible C3H/HeJ mice orally immunized with recombinant 29 kDa cysteine-rich and EHSALJC1 peptides. In agreement with our previous report (21), trophozoites produced numerous lesions on the epithelial surface, attracting an inflammatory infiltrate reminiscent of the human infection. Trophozoites could be detected in the cecal lumen and in the feces of mice for at least 20 days. Thus, C3H/HeJ mice intestinal infection provides a useful model to investigate many aspects of amebiasis including the evaluation of vaccine candidate antigens.

Microscopic analyses of cecal sections showed that non immunized infected mice developed the lesions described above including invasive trophozoites in the lamina propia (Fig. 5). In contrast, most of the mice that were orally immunized with the recombinant 29 kDa cysteine-rich peptide showed no lesions and inflammatory infiltrate. Mice immunized with recombinant EHSALJC1 peptide showed scarce lesions with moderate cellular infiltrate (Fig. 5). No trophozoites were found in the feces within both groups of the immunized mice. Thus, oral immunization with the two recombinant peptides successfully protected against intestinal infection in the mice, being the 29 kDa cysteine-rich protein the most effective. Protection appears to be associated with the production of sIgA antibodies detected in the feces of the immunized mice before challenge (Fig. 4). However, a high serum response, mainly IgG class, was also observed in both groups of immunized mice

after challenge before sacrifice, suggesting that local and serum responses are both important for the protection. Involvement of the serum antibodies in the protection against intestinal infection by *E. histolytica* has been also suggested in a study demonstrating a correlation between the serum response and a low incidence of amebiasis (12). Extensive protection studies with both recombinant peptides are currently in progress, including the evaluation of the cell-mediated immunity. Humoral and cellular immune responses have been suggested to contribute for protection in the studies using other recombinant antigens of *E. histolytica* (44,54).

Significant levels of sIgA against recombinant SREHP either expressed in a vaccine strain of *Salmonella typhimurium* or fused with the B subunit of CT were recently obtained in orally immunized mice and gerbils (46). Our findings support the suggestion of using amebic antigens as oral immunogens to induce protective local responses (46), although the mechanism of protection mediated by the secretory response remains to be characterized.

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Legends

Fig. 1. Nucleotide and deduced amino acid sequences of the novel *E. histolytica* cDNA clones isolated with human salivary IgA antibodies. Stop codons are marked with asterisks, except for Zsaljc1 which lacks it. (Figs. 11, 12 y 13 de la Tesis)

Fig. 2. Alignments of the deduced amino acid sequences of *E. histolytica* ribosomal protein- L23A and cyclophilin with homologue proteins. A. Ribosomal proteins of *E. histolytica*: EhRP-L23a, clone Zsaha51 (accession number XXXXX); Human (U43701); Rat (X65228); and Tobacco (L18908). Amino acid residues common to all ribosomal L-23a proteins are boxed. The highly conserved motif KKAYVRL involved in the binding to rRNA is double boxed and the critical leucine residue is indicated by arrow. B. Cyclophilins of *E. histolytica*: EhCyP, clone Zsaha54 (accession number XXXXX); *Plasmodium falciparum*: Pfalcip (U10322); *Saccharomyces cerevisae* CypA: Yeast (X17505); Human CypA (X52851) and the consensus sequence for eukaryotic cyclophilins. Conserved amino acid residues common to all cyclophilins are boxed; those relevant for the binding to cyclosporine A are indicated with dots. The tryptophane residue essential for the binding is marked by the arrow. (Figs. 11 y 12 de Tesis)

Fig. 3. Recombinant expression of EHSALJC1 and 29 kDa cysteine-rich peptides and their recognition by human salivary IgA antibodies. A. Expression of EHSALJC1 (lanes 1-3) and 29 kDa cysteine-rich (lanes 4-6) peptides in *E. coli* XL1-Blue(pRSET-Zsaljc1) and XL1-Blue(pRSET-Zsp1), respectively. Bacterial lysates were resolved on 12% SDS-PAGE applying 15 μg of protein to each lane. Lanes 1 and 4: lysates of recombinant cells after 5 hr

of induction. Lanes 2 and 5: lysates of uninduced recombinant bacteria; lanes 3 and 6: lysates of non transformed XL1-Blue cells. Induced proteins of 24 kDa (lane 1) and 29 kDa (lane 4) are indicated with arrowheads. B. Western blot of the metal-affinity purified recombinant 29 kDa cysteine-rich (lanes 1, 3 and 5) and EHSALJC1 (lanes 2, 4 and 6) peptides with saliva from an asymptomatic cyst carrier (lanes 1 and 2), pooled saliva from patients with intestinal amebiasis (lanes 3 and 4) and saliva from a patient with amebic liver abscess (lanes 5 and 6). Peroxidase-coupled goat anti-human IgA antibody was used as second antibody. (Fig. 16 de Tesis)

Fig. 4. Secretory and systemic responses against trophozoite lysate in C3H/HeJ mice orally immunized with recombinant 29 kDa cysteine-rich and EHSALJC1 peptides. Antibody responses were assayed by ELISA in sera on the 48th day (grey bars) and supernatants of feces from mice after 25 (white bars) and 48 (black bars) days of initial immunization. Bars represent the arithmetic mean ± standard deviation of the anti-ameba titers. (Fig 17 de Tesis)

Fig. 5. Light micrographs of cecal tissue sections from immunized and non-immunized mice challenged with *E. histolytica* trophozoites. Extensive infiltrate of neutrophils and lymphocytes beneath the mucosal epithelium (arrow) in the cecum from a non-immunized mice (A). Higher magnification of the infiltrate showing an active trophozoite (arrow) surrounded by cell infiltrate (B). No infiltrate is observed in the cecum from a mouse immunized with recombinant 29 kDa cysteine-rich peptide (C). Moderate cellular infiltrate in the cecum from a mouse immunized with recombinant EHSALJC1 (D). The preparations were stained with hematoxylin-eosin. Abreviation: e: epithelium.
(Fig. 20 de la Tesis)

Table I. Cloned antigens recognized with human salivary IgA antibodies.

Saliva source used in the screening	cDNA clone	Protein identity	Reference
Patients with intestinal amebiasis	ZSP1	29 kDa cysteine-rich protein	49
	ZSP6 and ZSP7	Serine-rich *E. histolytica* protein (SREHP)	47
	ZSP8	1-α elongation factor	13
Patient with amebic liver abscess	Zsaha1	Enolase	3
	Zsaha2	70 kDa heat shock protein	35
	Zsaha3	Acetaldehyde/alcohol dehydrogenase	6
	Zsaha4	Cysteine proteinase	41
	Zsaha51	Ribosomal L-23a protein (EhRP-L23a)	This work
	Zsaha54	Cyclophilin (EhCyP)	This work
Asymptomatic cyst carrier	Zsaljc1	Unidentified glutamic acid-rich protein (EHSALJC1)	This work

Effect of zinc on *Entamoeba histolytica* pathogenesis.

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ABSTRACT

The effects of zinc on *Entamoeba histolytica* activity were studied and wheter these effects caused modifications to its pathogenesis. Metal activity was evaluated *in vitro* regarding parasite viability, replication and adhesion to epithelial cells, and *in vivo* on pathogenesis. The results obtained *in vitro* show that zinc at concentrations from 0.01 to 1.0 mM does not affect amebic viability; however, it does decrease amebic replication and adhesion ($p< 0.01$ to 0.001, respectively). *In vivo* studies, performed on a model of experimental liver abscess in the hamster, indicate that the intra-peritoneal administration of a dose of zinc, 48 hours after the intrahepatic inoculation of amebic trophozoites, significantly inhibits ($p<0.001$) abscess development. Results indicate that zinc alters the functionality of the ameba *in vitro*, which is reflected by a decrease in replication and adhesion, and, *in vivo*, it is manifested by inhibition of its pathogenesis.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Entamoeba histolytica* ; ameba; zinc;amebic chemotherapy; BSA,bovine serum albumin; PBS phosphate-buffered saline; PMSF phenylmetilsulphonyl fluoride; PHMB p-hidroximercurybenzoate.

Enviado a Experimental Parasitology

INTRODUCTION

Entamoeba histolytica is a widely disseminated protozoan parasite that prevails in countries with socioeconomic problems, which generate sanitary and dietetic habits in the population which facilitate optimal development of parasitic diseases. The ameba, in its invasive form, uses potent and highly versatile mechanisms, which interact continuously and synergistically to bring about the disease-causing effect. Some mechanisms attack and destroy host tissue to achieve amebic penetration (Talamás and Meza 1988; Petri 1991), while others increase or decrease the host's normal cellular activity and alter different phases of the host immune response (Salata and Martínez-Palomo 1986; Denis and Chadee 1988; Gutiérrez *et al.* 1997). However, the close interaction between these mechanisms which exists from the beginning of the process hinders their separate description.

Of the parasite's invasive mechanisms, adhesion is essential for the development of the disease. Amebic adhesion, mediated, among other molecules, by N-acetyl-D-galactosamine lectin, is followed by important molecular changes. After target cell contact with the ameba and before its destruction by the parasite, calcium increases irreversibly and remarkably in that cell, whereas in the parasite, the cation increases only discreetly (Petri 1991). Parasite adhesion to the target cell also depends on its receptors. *E. histolytica* microfilaments are responsible for the movement of trophozoites and for the expression of receptors in the cytoplasmic membrane; in this respect, it has been observed that alterations of the microfilaments produce variations in receptors for fibronectin (Talamás and Meza 1988), through which the parasite binds to tissue.

Cytolysis of the target cell adhered to the ameba depends on the adherent lectins and on cytoskeleton integrity, a process which culminates with the active participation of other essential elements, such as enzymes, lysosomal proteins, amebapore and calcium (Ravdin *et al.* 1980). Amebapore, the chanel-forming polypeptide, is probably responsible for cytolysis by amebic contact (Lynch 1982) and calcium, which acts as a cofactor in the binding of phospholipase to the membrane of the target cell, is also required for microfilament movement and in cellular channel-formation (Lynch 1982). On the other hand calcium antagonists can inhibit amebic vesicle enzyme exocytosis (Ravdin *et al.* 1985), which supports the importance of this element in the progression of the cytolytic event. Amebic cytolytic activity is inhibited by some substances that act during different phases of the process. Thus, calcium-dependent phospholipase inhibitors (hydrocortisone, quinacrine) (Long-Krug *et al.* 1985), proteinkinase inhibitors (sphingosine) (Petri 1991); lysosomal protein inhibitors (cytochalasin B

and D) (Ravdin *et al.* 1980); calcium intracellular antagonists (TMB-8) (Long-Krug *et al.* 1985); amebic calmodulin antagonists (bepridil), and calcium channel blockers in the target cell (verapamil) (Ravdin *et al.* 1982) have been described.

Zinc is an element that intervenes in several essential functions of our organism such as growth, fertility, nocturnal vision, scar tissue formation (Prasad 1976) and immunity (Berger and Skinner 1974; Dardenne *et al.* 1982; Allen *et al.* 1983; Petrie *et al.* 1996) The metal actively participates in numerous metabolic processes, including DNA, RNA and protein synthesis (Williams and Loeb,1973). It stabilizes the structure of many proteins and specific sites of metal-protein interaction have been identified in some (Serrano *et al.* 1988). In the cytoskeleton, zinc exercises a direct effect on microfilaments (Krishna and Schwartz 1980) and in tubulin polymerization (Gaskin 1981; Serrano *et al.* 1988); aditionally, the metal has been suggested to competitively inhibit some effects of calcium (Wells 1973; Chvapil 1976; Baudier and Gerard 1990)).

Based on the above, we formulated the hypothesis that if zinc manages to interfere with the activity of calcium and/or alters the structure of the *E. histolytica* cytoskeleton, it probably affects its adhesion capacity as well as the cytolytic effect, and therefore its pathogenesis. This question and the expectation of participating in the search for new elements that contribute in the fight against amebiasis motivated the present study. The main objective was to determine the effect of zinc on the functionality of the *in vitro* ameba and, subsequently, on the development of *in vivo* amebic hepatic abscess. Results indicate that zinc alters amebic replication and adhesion *in vitro*, an effect that is reflected *in vivo* by experimental inhibition of the development of amebic liver abscess in the hamster.

MATERIAL AND METHODS

***E. histolytica* culture.** Pathogenic trophozoites (HM1-IMSS) of *E. histolytica* were axenically cultured in TYI-S-33 medium (Diamond 1961) supplemented with penicillin (100 UI/ml) and streptomycin (100 µg/ml) at 37°C.

Treatment of trophozoites with zinc. Trophozoites (10^5) in axenic Diamond medium (10 ml) were treated with different concentrations (0.001-10 mM) of zinc sulfate and incubated at 37°C for various periods. For their analysis, trophozoites were harvested at the desired times, placing the tubes in ice-cold water at 4°C and thereafter subjected to centrifugation at 150 x *g* for 5 min at 4°C. Amebae were subsequently washed three times in cold PBS (0.15 M, pH 7.4).

Determination of amebic viability. Effect of the metal on trophozoite viability was determined by microscopic observation and Neubauer chamber count with

the trypan blue exclusion technique, at 24, 48, 72 and 96 h of culture. Trophozoites placed in culture medium with 5.0 and 10 mM of zinc were observed at 0.5, 1.0 and 24 h, or, alternatively, with the purpose of studying the reversibility of the process, they were washed with PBS after remaining 30 min in contact with the zinc solution, and, subsequently, they were incubated again in Diamond medium without the cation,for the periods mentioned above.

Determination of amebic proliferation. It was evaluated by incorporation of tritiated thymidine (^{3}H-TdR). Cultures were marked 18 h before the harvest with 10 µCi of ^{3}H-TdR with high specific activity (25 Ci/mMol, Amersham, UK). Trophozoites were harvested by centrifugation at 150 x *g* for 5 min at 4°C and washed three times with PBS under the same conditions. The cell pellet was then resuspended in PBS and placed in 96-well polystyrene plates. Finally, the parasite was harvested (Cell Harvester, Brandel, Gaithersburg, MD) and radioactivity was determined by liquid scintillation counting (Packard Tri-carb) at 24, 48 and 72 h of culture. Results are expressed as average counts per minute (cpm) ± the standard error.

MDCK cell culture. MDCK cells were grown in Dulbecco's modified Eagle medium (GIBCO Laboratories, Life Technologies, Grand Island, N.Y.) at pH 6.8 supplemented with 5% fetal bovine serum, 100 UI/ml penicillin, 100 µg/ml streptomycin and 2.4% sodium bicarbonate, in culture flasks at 37°C, and in a 5% CO_2 humid atmosphere. 50,000 cells were cultured per well in 96-well polystyrene plates. Monolayers were washed with 0.15 M Na Cl solution and fixed with 0.25% glutaraldehyde for 10 min. Then, the plates were washed with a solution of 0.1 M glycine and finally with 0.15 M Na Cl and stored at 4°C until used (Kobiler and Mirelman 1981).

Study of amebic adhesion to monolayers of MDCK cells. The adhesion of normal amebic trophozoites or preincubated with 0.001 - 1.0 mM zinc during 72 h was studied by a microassay developed in our laboratory. The method is based on the ELISA technique in which we used a monoclonal antibody (C29) directed against one of the *E. histolytica* surface molecules (Ortiz-Ortiz *et al.* 1986). Optimal conditions for amebic adhesion to monolayers of MDCK cells in culture plates of 96 wells were the following: incubation of 5 x 10^4 trophozoites per microwell in 200 µl of 2.0 mM PBS, pH 5.7 by coincubating them for 15 min at 37°C with gentle shaking (75 rpm). The trophozoites adhered to the cells were then fixed with 0.25% glutaraldehyde in 0.15M NaCl, for 10 min at room temperature. Subsequently, trophozoites and cells were blocked with 200 µl of 1% BSA for 1 h at room temperature. The plates were washed with PBS containing 0.05% Tween 20 and 1.0% BSA at pH 7.4 (PBS-T-BSA). Next 50 µl of biotinylated monoclonal anti *E. histolytica* antibody to a surface molecule of *E. histolytica* diluted 1:400 was added and the plates were incubated for 1 h at 37°C. The plates were washed as described above and incubated with

streptavidin-peroxidase conjugate (1:2,000) for 1 h at 37°C; the preparation was washed again and, finally, o-phenylenediamine was used as a substrate. The reaction was stopped with 2.5 N H_2SO_4 and the optical density (OD) was measured at 490 nm in an enzyme-linked immunosorbent assay (ELISA) Processor M. (Marburg, Germany). A positive correlation was found between the number of amebae binding to the MDCK cells and the OD readings at 490 nm (Carrero *et al.* 1994).

Amebic antigen preparation. Amebic trophozoites of 72 hours of culture, normal and treated with zinc, were placed for their harvest at 4°C for 10 minutes and centrifuged at 150 x *g* for 5 min. The trophozoites were washed three times with PBS 0.14 M, pH 7.4 and were lysed by freeze and thaw cycles in presence of 1 mM of PMSF and 2 mM of PHMB as protease inhibitors. Total protein concentration was determined (Lowry *et al.* 1951).

Inoculation of hamsters with zinc solutions. In preliminary assays (data not shown) the concentrations and frequency of zinc solutions to be administered to the golden hamster (*Mesocricetus aureatus)* were defined. Animals (females, weighing 65 ± 5g, aged 7 to 8 weeks) were intraperitoneally inoculated with zinc sulfate solution at concentrations of 4mg/kg/day. One group (n=17) received 11 doses, 3 of which were administered 72, 48 and 24 h before the *E. histolytica* intrahepatic challenge (day 0); another group (n=10) received a single dose administered 48 h after the amebic challenge, and a third group (n=17), a control group, did not receive zinc but was challenged with amebae. Three other control groups which were not challenged with amebae were also included: the first received 11 doses of zinc, the second, 1 dose of zinc and the third received isotonic saline solution. Blood and liver samples were obtained from each group for the determination of anti-*E. histolytica* antibodies and the histopathologic study, respectively.

Induction of the experimental liver abscess. It was performed in hamsters according to the method previously described (Tsutsumi 1984). In brief, $1.5x10^6$ viable trophozoites in the logarithmic phase were intrahepatically inoculated into anesthetized animals. Twelve days after inoculation, animals were bled by cardiac puncture and subsequently sacrificed. The livers were extracted and the percentage ratio of the abscess size to healthy tissue was determined.

Tissue collection and processing. The livers obtained from the experimental animals were fixed in 10% formaldehyde in PBS. After 48 h fixation, tissues were washed and stored in PBS. Of the tissues dehydrated in alcohol gradients and included in paraffin, 4 μ were obtained and stained with eosin (Shehan and Hrapchak 1980) and Grocott-methenamine (Brinn 1983).

Anti-E.histolytica antibody determinations. These were performed by ELISA. In brief, the wells of the polystyrene microplates (Immulon 2; Dynatech Laboratories, Virginia, USA) were covered with lysed amebic trophozoites (2µg/well) in pH 9.6 carbonate buffer; incubated overnight at 4°C and blocked with 1% BSA, for 1 h at 37°C. The plasma obtained from treated animals and from the control group was diluted 1:100. After incubating for 2 h at 37°C and washing, anti-hamster peroxidase conjugated rabbit IgG (Accurate, San Diego CA, USA) was added diluted 1:1,000, incubation and washing procedures were repeated. The antigen-antibody reaction was revealed with orthophenylendiamine (Sigma) and read at 490 nm in a Bio-Rad 3550 scanner.

Statistical analysis. Variance homogeneity among groups under study was determined by Fisher's test. Significance of the differences was calculated with the Mann-Whitney test when variance was heterogeneous (Mann and Whitney 1947) or by Student's t-test when variance was homogeneous (Gosset 1908).

RESULTS

Effect of zinc on *E. histolytica* trophozoite viability. Viability of trophozoites treated with zinc at 0.01 to 1.0 mM concentrations did not show significant differences in relation to non-treated trophozoites. On the other hand, 5 and 10mM zinc had an almost immediate lethal effect, mortality being >50% at 30 min, >74% at 2h and >93% at 24 h; in the same intervals non-treated trophozoites presented mortality rates <10% by 2 h and <20% at 24 h (Fig. 1a). However, the lethal effect of the metal was inhibited when trophozoites treated with 10 mM zinc for 30 min were washed and cultured in Diamond medium without the cation for 72 h (Fig. 1b).

Effect of zinc on E. histolytica trophozoite proliferation.
The incorporation of ^{3}H-TdR (cpm) by zinc-treated and non-treated trophozoites was similar during the first 24 h, showing a significant decrease ($p<0.01$) at 48 h in the zinc-treated group. However, at 72 h only cultures treated with 1.0 mM zinc concentrations maintained their inhibitory effect ($p<0.001$) on parasite DNA synthesis (Fig. 2).

Effect of zinc on amebic adhesion. Trophozoite adhesion to MDCK cell monolayers was discreetly inhibited (10%) by treatment with 0.001 mM zinc. Nonetheless, when parasites were incubated with greater zinc concentrations (0.01, 0.1 or 1.0 mM) adhesion was significantly reduced ($p<0.001$) compared to that observed in non-treated trophozoites (Fig. 3).

Effect of zinc on liver abscess induction in hamsters. The development of amebic liver abscess is significantly inhibited in animals to which zinc is

administered intraperitoneally (one dose, $p<0.001$; 11 doses, $p<0.01$). Inhibition was greater with one dosis of the metal: only one of the ten animals tested developed an abscess of 20% of the liver; the nine remaining animals showed an abscess < 2% of the liver, and three of these exhibited total inhibition of the abscess. Hamsters which were administered 11 doses of zinc showed an abscess:liver tissue relationship lower than 20% in 11 animals, of 20% in 2 and from 50 to 65% in 2. Controls challenged only with amebae developed an abscess which involved from 40 to 75% of the liver (Fig. 4). We did not include in this group the data obtained from 5 animals who died as a consequence of the abscess before the end of the experiment. Mortality as a result of amebic liver abscess was null in animals who received zinc during the experiment.
On the other hand, hamsters in the control group which were not challenged with amebic trophozoites and received 1 or 11 doses of zinc did not show clinical disorders attributable to the administration of the metal along the experiment, nor macroscopic or microscopic alterations of the liver.

Histological studies. Animals protected by the administration of zinc, that is, with a macroscopically healthy liver showed either no evidence of alterations or it was minimal, such as centrolobulillar congestion and scarce lymphocytic infiltration (Fig. 5a). Animals partially protected with zinc developed small liver abscesses which microscopically showed areas of necrosis with interiorized trophozoites, lymphoplasmocytic infiltrate and occasionally lymphoid cummulates surrounding microabscesses. This pattern, if magnified, i.e., confluence of microabscesses, extense necrosis, numerous trophozoites, accompanied of large amounts of polymorphonuclear leukocytes were observed in control animals which were only challenged with amebae (Fig. 5b). The histologic study of macroscopically healthy livers obtained from normal animal controls and from animals which only received zinc presented hepatic tissue of normal characteristics (data not shown).

Effect of zinc on the humoral response of hamsters intrahepatically inoculated with *E. histolytica*. Animals infected with amebae and treated with 1 or 11 doses of zinc presented similar anti-*E. histolytica* antibody response (0.829 ± 0.28 and 0.805 ± 0.47 respectively). Furthermore, animals treated or no treated with zinc, but with abscesses larger than 40% of the liver showed higher values (1.014 ± 0.18) although the difference was not significant ($p>0.05$).

DISCUSSION

Results presented indicate that 0.01 to 0.1mM zinc diminishes *E. histolytica* replication and adherence *in vitro*, which are reflected *in vivo* (by administration of 4mg of zinc sulfate) as the inhibition of the development of liver abscess after intrahepatic challenge with viable trophozoites. Growth of amebae at 72 h with doses which did not affect their viability (<1.0mM) was significantly low ($p<0.001$)

than that observed in amebae without the metal. With greater doses (5 and 10 mM) mortality was evident after 30 min (50%), however, this effect was inhibited when trophozoites were washed within the mentioned period, and regrown in Diamond medium without the ion. This inhibition could be due to constant metal exchange or to its reversible binding.
Zinc has great affinity for histidine and cysteine (O'Dell 1992; Berg 1990). and the latter amino acid is present in a 29 kDa protein named histolysine, which is expressed on the surface of the ameba (Osorio *et al.*1992). Zinc could remain on the surface and generate modifications directly on the membrane and/or, through signal emission, toward the interior of the trophozoite. Another possibility is that zinc either diffuses or is transported into the parasite bound to cysteine as occurrs in the intestine (Hempe and Cousins 1991). Once the zinc is inside, it can interact with nucleic acids indirectly keeping contact among the proteins that contain the metal and which bind to DNA, or else, directly as do some cells in the so-called "zinc finger" domains (Hempe and Cousins 1991; Osorio *et al.* 1992; Freemont 1993). Additionally, the effect of zinc on proteins similar to those of the parasite (Ratka 1989) and on calcium which it antagonizes or removes from its binding sites (Baudier and Gerard 1990) must be recalled.
Alterations of the cytoskeleton structure besides its possible effect on replication could originate modifications both in form and movement, such as exocytosis, and the expression of amebic receptors. These latter factors are responsible both of the transmission of signals into the interior of the parasite and of the adherence to the target cell. Thus, if these were the events mainly affected by the metal, it could be sufficient to limit the tissue penetration capacity of the parasite. At present, we are studying the effect of zinc on the structure and function of the amebic cytoskeleton, as well as the effect it has on calcium, an element which besides being necessary for amebapore activity and parasite phospholipase, participates in the dynamics of cytoskeleton proteins and in cytotoxicity and lysis of the target cell (Lynch *et al.* 1982).

The deletereous effect that zinc has *in vitro* on the amebic functions of replication and adherence may alter its virulence, which is manifested *in vivo*, since zinc inhibits the formation of liver abscesses in hamsters challenged with *E. histolytica*. This effect was observed in the two groups which received the metal, alhough results were better in animals receiving one sole dose of zinc (Fig. 4). Side-effects attributable to the administration of zinc were not detected in any case.

No significant differences in the levels of antiamebic antibodies were observed between animals protected with 1 or with 11 doses of zinc. However, the most elevated levels of antibodies were observed in animals which developed amebic liver abscess which affected the liver in more than 40%. This is probably the result of the persistence and development of amebae in the liver which generates a constant antigenic stimulus.

Zinc may favour the control of the amebic invasion through its participation in some of the stages which intervene in the parasite inactivation process, the induction of a host immune response or in the protection of the target cell. In animals which received 11 doses of zinc (3 previous to the amebic challenge) arrival of the metal could have exerted on amebae one or some of the effects observed *in vitro* (replication and adherence inhibition) which could inactivate the parasite, acting either alone or combined. However, this does not totally explain the observations in animals who received only one dose of zinc after amebic challenge, since adherence or replication inhibition 48h after amebic invasion was initiated could help, in the best of cases, to hinder amebic extension but could not revert the process. Therefore, we suppose that other mechanisms are involved to to achieve this effect.

Zinc increases alpha-TNF and IL-I production and secretion in mononuclear phagocytes (Scuderi 1990) and in low concentrations it is capable of stimulating monocyte and lymphocyte activity, both *in vivo* and *in vitro* (Berger and Skinner 1974; Kirschner and Salas 1987; Scuderi 1990). On the other hand, when diminished serum levels of the metal are maintained for periods longer than two weeks, as well as in very intense zinc deficiency, the opposite succeed thus the cellular activity mentioned above is reduced. This was observed in a previous study (Vega *et al.* 1994) on patients with liver cirrhosis produced by alcohol. In them, circulating zinc values (50+3.3 µg/dl) were lower by up to 40% than those detected in the normal adult population (86+4.5 µg/dl) and patient lymphocyte response to various mitogens was frankly reduced. Interestingly, in healthy humans it has been observed that zinc ingested in amounts five times greater than normal doses during more than 8 days produces a reduction in chemotaxis and in phagocytosis (Prasad *et al.* 1988; Blazseck and Mathe 1984; Chandra 1984); further on (15 days) cell proliferation diminishes too (Chandra 1984).

Zinc can also bind to some proteins which link and/or transport calcium (Csermely 1989; Waisman *et al.* 1990; Picello *et al.* 1992). Occasionally these show more affinity for zinc than for calcium (Serrano *et al.* 1988) and can have binding sites for zinc (Csermely 1989; Picello *et al* 1992). The amebapore induces an increase in the host cell calcium levels (Lynch *et al* 1982) and the generation of voltage currents (N, L, T) which are activated by calcium and can be blocked by zinc (Winegar 1990; Buselberg *et al.* 1992). Thus, zinc could keep the calcium channels ionically blocked for longer, which would prevent cell lysis.

In accordance with the inhibitory effect of zinc on hepatic abscess formation observed in this study, we can infer that the stimulus which triggers some of the defensive mechanisms, which under normal conditions are incapable of stopping the amebic infection, was produced in animals with a single dosis of zinc 48 h after amebic challenge. In animals which received 11 doses of zinc, the stimulus may have been excessive causing a depression of the immune response. This inhibition would favour amebic tissue invasion which is reflected in greater

number of miniabscesses and in the larger size of the abscess developed by four animals of this group (Fig. 4). Work is in progress to determine the effects of zinc on cell-mediated immunity of experimental animals infected with E. *histolytica*.

It is impossible not to speculate on the utility of a single dose of zinc exempt of side-effects to control amebic diseases in humans. Zinc may not function as an antiamebic drug on its own, but the possibility should be considered that it can assist other drugs used in treatment or help in immunological manipulations which confer protection against amebic diseases, and preventing some other pathological processes of the host.

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FIGURE LEGENDS

Figure 1. Effect of zinc on *E. histolytica* viability. a) Growth kinetics of non-treated *E. histolytica* trophozoites (□) and treated with 5.0 (♦) and 10.0 (O) zinc. Viability was determined at 24, 48 and 72 h by trophozoite count using the trypan blue exclusion technique. b) Reversion of the effect of zinc. Viability as mentioned above, was evaluated in non-treated *E. histolytica* trophozoites (□),

and in treated with 10.0 mM for 24 h (O) or 30 min (●); at the last time, amebae were washed with PBS, placed in TYI-S-33 and reincubated for 72 h.

Figure 2. *E. histolytica* trophozoite incorporation of ^{3}H-TdR. Capture (cpm) of high specific activity ^{3}H-TdR was determined with a liquid scintillation counter innon treated (□) and zinc treated (O- 1.0, Δ- 0.1, ■- 0.01, ▲- 0.001 mM) parasites cultured for 24 48 and 72 h.

Figure 3. Action of zinc on adherence of amebic trophozoites. A microassay based on the ELISA technique in which a monoclonal anti-*E. histolytica* antibody was employed, served to determine adherence of trophozoites incubated for 72 h in culture medium without and with zinc (0.001-0.1mM) to monolayers of MDCK cells.

Figure 4. Amebic liver abscess. Groups of hamsters were intrahepatically inoculated with viable amebic trophozoites. Two of them received intraperitoneal doses of ZnSO4 (4mg/kg): 11 doses (3 previous to and 8 after the chalenge) or 1 dose (48 h after de chalenge). The third group only received 11 doses of physiological saline solution. All animals were sacrificed on day 12. The liver was extracted and the percentage relationship between the weight of the abscess and healthy liver tissue was determined (for more details see the text)

Figure 5. Histopathologic study of hamster liver inoculated with *E. histolytica* trophozoites. a) Representative liver tissue of hamsters treated with zinc (1 or 11 doses) that did not develop abscess. b) Representative liver tissue of hamsters not treated (controls) with zinc and which developed amebic liver abscess.

SECRETORY IMMUNE RESPONSE IN PATIENTS WITH INTESTINAL AMEBIASIS

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Running head: Secretory IgA response during intestinal amebiasis

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ABSTRACT

The secretory immune response in saliva from intestinal amebiasis patients against an antigen obtained from *Entamoeba histolytica* membrane was studied. Western blot analysis indicated that patient saliva contains secretory IgA antibodies against antigens with molecular masses ranging from 170 to 24 kDa, part of which are also recognized by saliva from healthy subjects. However, antigens of 170, 125, 46 and 37 kDa are recognized more frequently (>90%) by the secretory IgA from patients with intestinal amebiasis than by that from healthy subjects (<10%).

P.D. Enviado a Infection and Immunity

Amebiasis produced by *Entamoeba histolytica* constitutes a serious public health problem which causes 50 million cases of colitis and extraintestinal abscesses yearly and about 50,000 deaths, ranking third worlwide among parasitic causes of death (32,33). The parasite colonizes the intestine where *E. histolytica* adherence to cells appears to preced tissue invasion (the colon) or tissue lysis (the liver) (25). Since the intestinal mucosa is the most important barrier against the parasite and IgA the immunoglobulin which predominates in the mucosas (20), we decided to study the presence of anti-*E. histolytica* secretory IgA (sIgA) antibodies in saliva from patients with intestinal amebiasis. Although, several studies have demonstrated the sIgA antibody response to *E. histolytica* (1,2,8,10,14,16,23,27), little is known about the parasite antigens recognized by this immunoglobulin during amebic infection in humans. On the other hand, since surface antigens are mainly the first to be recognized by the host, we decided to use *E. histolytica* membrane extracts to identify the antigens most frequently recognized by the sIgA anti-ameba antibodies present in the saliva of infected patients. These may be helpful in the develpment of better diagnostic methods and also in the identification of potential candidates for the design of a vaccine. The study was performed using an immunoblot (31) assay and the results were analyzed by immunoplot (19).

Non-stimulated saliva samples were collected from 20 patients with a diagnosis of intestinal amebiasis confirmed by sigmoidoscopy and stool examination. These saliva samples were kindly provided by Dr. Edmundo Godinez from the Hospital General de México. Control samples were obtained from 19 individuals with no clinical symptoms of amebiasis living in México. The stool examination (three samples, obtained on three consecutive days) of this group was negative, indicating the absence of amebiasis. Saliva was collected as previously described (8). Briefly, each individual was asked to dribble into a disposable funnel from which approximately 5 ml of whole saliva were collected into a test tube immersed in ice. The saliva was centrifuged at 10,000 X *g* for 30 min, and the supernatant frozen at -20°C until used.

Axenic cultures of *E. histolytica*, HM1:IMSS strain, were grown in TYI-S-33 medium with 100 U of penicilin per ml-100 µg of streptomycin sulfate per ml-0.25 µg of amphotericin B per ml at 37°C in culture flasks (12). Trophozoites were harvested after 72 h of growth by chilling the flasks on ice for 10 min and centrifuging at 150 X *g* for 10 min at 4°C. The parasites were then washed three times with phosphate-buffered saline (PBS), pH 7.4, by centrifugation as described above. The viability of parasites was assayed by trypan blue exclusion and it was about 91%.

A suspension of trophozoites (10^7) was prepared in PBS containing protease inhibitors, namely : 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM *p*-hydroximercuribenzoate (PHMB), 2 mM iodoacetamide (IAA) (Sigma Chemical Co., St. Louis, MO) and used for electrophoretic studies. Trophozoite membranes were obtained as in Aley *et al.* (3) with slight modifications. All steps were performed at 4°C. In brief, washed trophozoites were agglutinated with concanavalin A (1 mg/ml, Sigma), sedimented by centrifugation at 50 X g for 1 min and resuspended in 10 mM Tris-HCl, pH 7.5 containing 2 mM PMSF, 1mM PHMB, 2 mM IAA and 1 mM $MgCl_2$. After 10 min in this buffer, trophozoites were homogenized by 10 strokes in an all-glass Dounce homogenizer with a tight-fitting pestle (Wheaton Scientific, Millville, N.J.). Subcellular fractions were isolated by centrifugation over 0.5 M mannitol/0.58 M sucrose and 20% sucrose gradients. Plasma membranes were resuspended in 10 mM Tris-HCl, pH 7.5 containing 2 mM PMSF and 1 mM PHMB. Protein content of these fractions was determined by the method of Bradford (6) with reagents from Bio-Rad Laboratories (Richmond, Calif.) and bovine serum albumin (BSA, Sigma) as the protein standard. All samples were stored frozen at -70°C.

Extracts of whole or membrane trophozoites from *E. histolytica* were boiled in SDS-PAGE sample buffer and electrophoresed in 10% SDS-PAGE gels prepared essentially according to Laemmli (18). Molecular mass standards (Sigma) of 191, 117, 91.8, 72.7, 57.8, 40.8, and 34.1 kDa were used. After SDS-PAGE, proteins were electrophoretically transferred from the polyacrylamide gels to nitrocellulose membranes (Bio-Rad) as described by Towbin *et al.* (31) using a GENIE electrophoretic blotter (Idea Scientific Co., Corvallis, Or.). Transfer was allowed to proceed for 1 h and the blotting buffer was 25 mM Tris and 192 mM glycine in 20% (v/v) methanol. After transfer, the nitrocellulose membrane was treated with 3% BSA in PBS containing 0.3% Tween 20 (PBS-Tw) (Sigma) and then cut into strips which were washed and incubated overnight at 4°C with the patient or control saliva under investigation. On the next day, the strips were washed five times with PBS-Tw and exposed overnight to peroxidase-conjugated affinity-purified goat anti-human IgA (α-chain specific, Sigma) diluted in PBS-Tw with BSA. After washing as above, the strips were incubated with 3,3'diaminobenzidine tetrahydrochloride (Sigma) upon addition of 30 µl H_2O_2 in 10 ml of 0.1 M Tris buffer, pH 8.3, for 10-20 min until dark bands appeared. Finally, the reaction was stopped by washing the strips with distilled water. Bands obtained by immunoblotting were digitalized and processed by Gel Scan (K.M. Allaim, M.L. Metcker, Department of Molecular and Human Genetics, Baylor College, Houston, TX). The relative mass (M_r) of relevant antigens was calculated according to their R_f. From the obtained data, the most frequently recognized bands were selected, and an immunoplot (19) was prepared.

The immunoplot method described by Larralde *et al.* (19) was used to determine the frequency of each *E. histolytica* antigenic fraction with the saliva of infected individuals. The data obtained from the Western blots was plotted against the frequency of reactivity of the same antigenic fraction with the saliva of healthy individuals. The frequency value for each particular antigenic fraction was obtained, taking as reference a pattern previously standardized that contains all the antigenic bands recognized and ordered according its M_r.

When submitted to SDS-PAGE analyses, total *E. histolytica* extracts showed very complex patterns. The densitogram revealed approximately 30 bands with M_r ranging from 210 to 14 kDa. On the other hand, the membrane extract showed approximately 23 bands with M_r ranging from 210 to 18 kDa. Among them the following polypeptides stand out: 170, 155, 125, 96, 76, 62, 53, 48, 37, 30, 24, 22 y 18 kDa (Fig. 1). In subsequent studies we only used the membrane extract due not only to its lesser complexity but also to its possible relevance as a recognition target of the host immune system. Immunoblot studies performed in saliva from patients with amebiasis and from healthy subjects showed that both groups recognized antigen with M_r from 170 to 24 kDa, although generally, intensity and recognition frequency of the membrane antigens was greater with the saliva from infected subjects. However, antigens of 170, 125, 96, 46 y 37 stand out since they are preferentially recognized by sIgA from pateints with amebiasis (Fig. 2).

The reactivity of normal and patient salivas against the membrane antigens was analyzed using simple immunoplot (19). There are many antigens particularly immunogenic to the patients, as can be seen in the upper left quadrant, where at least proteins of 170, 125, 96, 90, 46, and 37 kDa are recognized mainly by the saliva from amebic patients at a frequency >65%. Among these antigens, that of 37 kDa stands out which was recognized by all sIgAs of patients with intestinal amebiasis and reacted with only one of the healthy subject salivas. Other antigens recognized by all patients with amebiasis are those of 62, 53, 48, 42 and 24 kDa, although they also react with >21% of the saliva from healthy subjects. Antigens recognized mainly by patients, albeit with a low frequency, are located in the lower left quadrant, over and close to the y-axis. Among them we have the 92 and 68 kDa which react with patient saliva with a frequency of ≤50% and are detected by the saliva of healthy subjects with a frequency of 15%. Antigens from the membrane preparation recognized frequently by both healthy subject and patient saliva are located in the upper right quadrant of the immunoplot, among them, those of 84 and 76 kDa. The lower right quadrant, which is empty, corresponds to antigens that could be recognized mainly by normal saliva.

We herein report the secretory immune response of patients with intestinal amebiasis. Saliva from these subjects presents anti-*E. histolytica* sIgA antibodies which recognize 15 different antigens from a membrane extract. These can be classified into three groups. The first group includes those which react preferentially with the sIgA of patients with intestinal amebiasis and occasionally with that of healthy subjects (170, 125, 96, 90, 46 y 37 kDa). It also includes those antigens which react chiefly with patient sIgA (100%) and also react with healthy subject sIgA with certain frequency (>21%) (62, 53, 48, 42 and 24 kDa). In this first group, the antigen of 37 kDa stands out which is recognized by sIgA of all patients with intestinal amebiasis here studied and only by 5.2% of the healthy subject sIgA. In the second group, we find the antigens recognized by patient sIgA with moderate frequency (≤50%) and by healthy subjects with low frequency (15%) (92 and 68 kDa). Finally, the third group is formed by antigens recognized with high frequency by amebiasis patients and by healthy subjects (85%) (84 y 76 kDa).

Previous investigations have reported the presence of anti-*E. histolytica* in bile, maternal milk, collostrum, intestinal fluids and saliva (1,2,8,10,14,16,23,27). However, the secretory immune response has not been characterized. In this report we tried to do this using saliva from amebiasis patients and a membrane antigen. *E. histolytica* membrane antigens are very likely the first to be recognized by the immunologic system of the infected individual and are possibly the most relevant. The role of sIgA in amebiasis has been stated previously. Thus, in our laboratory, Carrero *et al.* (8) described the property of sIgA from amebiasis patients to inhibit *E. histolytica* adherence to epithelial cells *in vitro*. This sIgA is directed in part to the galactose-binding lectin of 170 kDa. Recently, Kelsall y Ravdin (17) went further by demonstrating that immunization of rats with the 260 kDa *E. histolytica* galactose-inhibitable lectin elicits and intestinal secretory IgA response that has *in vitro* adherence-inhibitory activity. In addition, a recombinant cystein-rich section of this lectin elicits an intestinal sIgA response that inhibited the galactose-specific adherence of axenic trophozoites to Chinese hamster ovary cells (5). More recently, Zhang *et al.* (34) informed gerbil protection against amebic liver abscess, the most common extraintestinal complication of amebiasis by oral immunization with an attenuated vaccine strain of *Salmonella typhimurium* expressing another ameba antigen, the serine-rich *E. histolytica* protein. The immune animals produced mucosal IgA and serum IgG anti-amebic antibodies. Thus, it is evident the importance of sIgA in protection against amebic infection.

Several reports exist on characterization of the immune response in the sera of individuals with invasive amebiasis. Most studied sera recognize proteins of 170, 125, 96, 90, 59 y 37 kDa (13,15,16,26,29). Thus, Joyce and Ravdin (15) report that antigens of 90, 59 and 37 kDa are recognized most frequently by serum from patients with amebic liver abscess (ALA). Similar studies

with patient sera identify various antigens, among them of 88 y 37 kDa (4), the first of which could correspond to the 90 kDa antigen here described. Additionally, 90% of ALA patient sera exhibited very high antibody titres and a group of them strongly identified two peptides, of 96 and 130 kDa, present on the trophozoite surface (21). Likewise, we have found that 100% of ALA patient sera recognize various antigens, among them those of 167, 97, and 37 kDa and most of them reveal the presence of a peptide of 48 kDa (11). These antigens could correspond to those of 170, 125, 96, 46 and/or 48 and 37 kDa reported herein, and also present in most patients with invasive amebiasis.

Some biological properties are known of certain antigens recognized by the patient sIgA here studied. The 37 kDa peptide, which seems relevant in the recognition of intestinal amebiasis patient sIgA, has been described as a possible fibronectin receptor (28). Specific binding and degradation of fibronectin performed by HM1 trophozoites, except in the case of *E. histolytica*-like Laredo, indicate that invasive amebiasis respond to the binding of this ligand by liberating the proteases necessary to degrade it. The presence of antigens of 170, 125 and 96 kDa in pathogenic and non-pathogenic strains has been reported (13,24,29); consequently, the recognition of these antigens by healthy subject saliva may be due to a previous exposure to *Entamoeba* of pathogenic or non-pathogenic species. We do not know if the same can be applied to the rest of antigens, since no information exists as to their presence or abscence in other strains. On the other hand, the presence of sIgA against amebic antigens which could be characteristic of pathogenic strains, in healthy individuals, may signify persistance of antibodies after self-limited infections with pathogenic *E. histolytica* (26). It is worth mentioning that the saliva samples used in the present study were obtained from patients and controls from a population where a high parasite load and a high risk of infection were expected (7). Under these conditions, intestinal parasites in contact with the host's tissue may elicit a secretory immune response even in the absence of invasiveness. It has been documented that both food antigens and nonviable bacteria may elicit a secretory immune response in healthy individuals (9,22). Therefore, a positive result in healthy subjects with a negative coproparasitoscopic result may signify recent contact.

In summary, the results here reported provide information on the secretory immune response to *E. histolytica* in a group of patients with intestinal amebiasis. The response indicates that sIgA antibodies against numerous membrane antigens of *E. histolytica* are present in the saliva of such patients and in most cases, in much lower frequency in healthy individuals. However, part of these sIgA antibodies recognized antigens of 170, 125, 46 and 37 kDa seldomly present in healthy individuals.

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FIGURE LEGENDS

Fig. 1. SDS-PAGE of *E. histolytica* antigens under non-reducing conditions: (A) molecular mass markers; (B) total antigen, and (C) membrane antigen. On the right side, the densitogram of the SDS-PAGE gels: membrane (----) and total (——) *E. histolytica* antigens.

Fig. 2. Immunoblot analysis of the membrane antigen from *E. histolytica* with saliva from patients with intestinal amebiasis.

Fig. 3. Immunoblot analysis of the membrane antigen from *E. histolytica* with saliva from healthy control individuals.

Fig. 4. Immunoplot of simple frequencies of the population studied: intestinal amebiasis (*y*-axis) *vs* healthy controls (*x*-axis). In the upper left quadrant are the proteins recognized mainly by the saliva from patients with intestinal amebiasis.