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INDICE

LISTA DE FIGURAS

CAPITULO II

CAPITULO III

CAPITULO IV

CAPITULO V

LISTA DE TABLAS

CAPITULO II

CAPITULO III

CAPITULO IV

RESUMEN

Aunque las enfermedades infecciosas causan un tercio de las muertes en todo el mundo, su impacto es aún mayor en los países subdesarrollados, donde son 30 veces más frecuentes. Las infecciones respiratorias agudas y las enfermedades diarréicas son las enfermedades infecciosas de mayor importancia global: a ellas se deben tres cuartas partes de las muertes por infecciones en el mundo y en México ocupan los primeros lugares como causas de muerte, sobre todo en niños. Estas enfermedades se deben a la colonización y daño de las mucosas —las cubiertas epiteliales húmedas que delimitan y recubren el interior de los aparatos respiratorio, digestivo y genitourinario, cuya superficie total es unas 200 veces mayor que la superficie de la piel— por agentes microbianos llamados patógenos adherentes.

La vacunación es uno de los medios más eficaces y baratos para prevenir las enfermedades infecciosas. Sin embargo, el desarrollo de nuevas vacunas que ayuden a prevenir las enfermedades de mayor incidencia en los países pobres está prácticamente paralizado porque el mercado de fármacos nuevos es mucho más lucrativo que el de las vacunas. Por otra parte, desde hace buen tiempo se sabe que la vacunación oral contra los patógenos adherentes es más simple e induce inmunidad protectora más intensa y duradera que la vacunación parenteral. Por eso sorprende que entre las vacunas más empleadas en todo el mundo contra enfermedades infecciosas prevenibles de esta manera, sólo dos —las del cólera y la polio— se administren por vía oral. Por eso resulta tan importante desarrollar nuevas vacunas orales efectivas como mejorar las parenterales que ya existen.

El desarrollo de vacunas orales eficaces contra los patógenos adherentes depende de avances en el conocimiento de dos áreas clave: 1) la inmunidad de las mucosas —las barreras que constituyen la primera y más extensa e importante línea de defensa contra los patógenos adherentes—, y 2) los mecanismos patogénicos de los agentes infecciosos, particularmente la identificación y caracterización de los factores moleculares que provocan las alteraciones estructurales y metabólicas en las enfermedades infecciosas y pudiesen servir como antígenos en las vacunas correspondientes (Publicado en *Avance y Perspectiva* 1995;14:75 y *Archives of Medical Research* 1994;25:253)

En este trabajo abordamos el estudio de los mecanismos patogénicos y la inmunidad de la mucosa intestinal en la amibiasis. La amibiasis intestinal es la forma más común de la enfermedad amibiana y la fuente de transmisión de *Entamoeba histolytica*, el protozoario parásito que la causa. El balance entre la virulencia de los trofozoítos de *E. histolytica* infectantes y la inmunidad local probablemente determina el grado de invasión a la mucosa intestinal por el parásito. Sin embargo, los actuales modelos animales de amibiasis intestinal tienen las desventajas de ser bastante laboriosos, tener baja reproducibilidad, y de que las lesiones que producen son sólo perceptibles varios días después de la inoculación intracecal del parásito

Para mi trabajo de tesis de maestría (de la que resultaron dos artículos publicados en *Experimental Parasitology* 1993;77:162 y *Archives of Medical Research* 1992;23:197), desarrollé un modelo *in vitro* de amibiasis intestinal, con el que encontré que la adición de lisados de trofozoítos de *E. histolytica* HM1 en el lado luminal de preparaciones de colon (mantenidas en medio basal para el cultivo de amibas o solución de Ringer) provoca una caída inmediata de la diferencia de potencial (DP) y la corriente de corto circuito (Icc), mientras que lisados derivados del mismo número de trofozoítos de *Entamoeba invadens* PZ (cepa virulenta para reptiles) no tienen ningún efecto. Los cambios microscópicos más tempranos de daño en los segmentos expuestos a los lisados de *E. histolytica* son vacuolas translúcidas ubicadas en la base del epitelio intestinal, seguidos por el desprendimiento de la capa epitelial de la lámina propia y la destrucción de células epiteliales interglandulares.

Para este trabajo de tesis doctoral empleé nuestro modelo experimental para: i) caracterizar los efectos electrofisiológicos y morfológicos a la mucosa intestinal de los lisados amibianos, ii) determinar la participación de las cisteín-proteasas amibianas en al virulencia y iii) caracterizar la respuesta inmune intestinal y determinar su papel protector *in vitro*. Cada una de estas tres partes generó una publicación en una revista internacional con arbitraje y un manuscrito recién enviado a publicación. A continuación describo los aspectos fundamentales de cada parte.

Efectos Electrofisiológicos y Morfológicos de los Lisados Amibianos

La magnitud de los efectos electrofisiológicos, principalmente sobre la DP y la resistencia eléctrica transepitelial (R), dependió de la dosis de lisados amibianos y correlacionó con el grado

de las lesiones microscópicas. La dosis más pequeña (25,000 trofozoítos lisados) indujo la formación de pequeñas vacuolas translúcidas que bajo el microscopio de luz se ubicaron entre la capa epitelial y la lámina propia; con el microscopio electrónico tales vacuolas se ubicaron intracelularmente bajo los núcleos, en la base de las células epiteliales. Con la dosis intermedia (50,000 trofozoítos lisados), la capa epitelial comenzó a separarse de la lámina propia y hubo necrosis incipiente, principalmente en las células epiteliales interglandulares; bajo el microscopio electrónico estas preparaciones tenían acortadas las células epiteliales con pérdida de uniones intercelulares, núcleos deformes y microvellosidades fragmentadas. La dosis más alta (100,000 trofozoítos lisados) ocasionó descamación masiva y destrucción de las células epiteliales interglandulares. También analizamos el curso temporal del proceso de daño. En preparaciones tratadas con la dosis intermedia de lisados, después de 15 min de exposición había incipiente vacuolizacion en la base de las células epiteliales. Después de 40 min, las vacuolas habían aumentado en tamaño y se habían perdido las uniones intercelulares, y estos hallazgos correlacionaron con la caída de la resistencia eléctrica. Después de 80 min, cuando los valores de DP e Icc habían llegado a ser nulos, había grandes vacuolas subnucleares en la base de las células epiteliales interglandulares, la pérdida de las uniones intercelulares era completa y había también destrución masiva de células epiteliales (Publicado en *Parasitology Research* 1993;79:517).

Las lesiones estructurales observadas en nuestro modelo son parecidas a las que se han observado en biopsias humanas y amibiasis intestinal experimental. Además hemos sido capaces de detectar las lesiones electrofisiológicas y morfológicas más tempranas que han sido descritas hasta ahora en la amibiasis intestinal experimental: caída de DP e Icc, y vacuolización en la base del epitelio. Nuestro modelo también tiene las siguientes ventajas sobre otros modelos *in vitro* y en animales: a) es más cercano a la amibiasis intestinal porque el blanco es un segmento intestinal con todas sus capas (desde la mucosa hasta la serosa) y no células en cultivo, b) las propiedades electrofisiológicas de los segmentos de colon expuestos a los trofozoítos lisados pueden correlacionarse con sus cambios morfológicos y bioquímicos, c) la ubicación inmediata de las lesiones microscópicas es posible porque la interación intestino-lisados tiene lugar en un área mínima y específica, d) los resultados son reproducibles, en contraste con los de animales

inoculados intracecalmente, que son inconsistentes y toman varios días para desarrollarse.

Participación de las Cisteín-Proteasas en la Virulencia Amibiana

Han sido propuestos varios factores potencialmente patogénicos de amibas, tales como proteinasas con actividad citotóxica, una proteína formadora de poros, y sustancias parecidas a ciertas neurohormonas. El daño y la degeneración de las células epiteliales intestinales indican que los factores histolíticos juegan un papel importante en la virulencia amibiana. El jerbo ha sido considerado como un modelo animal apropiado de amibiasis intestinal, caracterizado por lesiones mucosas microulcerativas, infiltrado inflamatorio, edema en la lámina propia asociada con focos necróticos, así como aumento en la producción de moco. Nosotros hemos encontrado que los lisados de trofozoítos de *E. histolytica* también causan una caída en los valores de de las propiedades electrofisiológicas en preparaciones de ciego de jerbo montadas en cámaras de Ussing.

Usando al jerbo como modelo animal, comparamos la enterotoxicidad y la actividad de cisteín-proteinasas (CP) de una cepa de *E. histolytica* HM1 de baja virulencia (que había sido mantenida en el laboratorio por más de 15 años) con la clona 1659 altamente virulenta (derivada de la cepa HM1 por pases sucesivos a través del hígado de hámster). La enterotoxicidad de 50,000 trofozoítos lisados se determinó sobre segmentos intestinales de jerbo montados en cámaras de Ussing; la actividad de CP de los lisados fue ensayada en geles de poliacrilamida-SDS copolimerizados con gelatina y con un sustrato sintético específico para CP, la carbobenzoxi-L-arginina-L-arginil-p-nitroanilina. El tratamiento de los segmentos de ciego de jerbo con lisados amibianos causó una caída inmediata de las propiedades electrofisiológicas (DP, Icc y R) del jerbo cuyas tasas de deterioro fueron mayores con lisados de la clona 1659 que de la cepa HM1. El suero humano no inmune e inmune y un inhibidor específico de CP llamado E-64 (trans-epoxisuccinil-L-leucilamido (4-guanidina)-butano) previnieron la caída de las propiedades electrofisiológicas. Las gelatinasas, menos activas en los trofozoítos HM1 que en los 1659, fueron mejor conservadas en lisados que contenían p-hidroximercuribenzoato (pHMB) 10 mM para prevenir la autoproteólisis: en lisados sin pHMB ninguna banda de gelatinasa fue observada en HM1, mientras que en 1659 observamos bandas intensas de 30 kDa, 35 kDa, 44 kDa y 75 kDa;

en lisados con pHMB únicamente encontramos bandas de 53 kDa y 75 kDa mucho más intensas que en 1659; la banda de 75 kDa fue apenas visible en HM1. La actividad de CP fue 17 veces más alta en lisados 1659 que en HM1; fue inhibida por E-64 (dosis inhibitoria media: 20 μM), fue estimulada por 2-mercaptoetanol (ME) 3.7 veces en HM1 y 2.4 veces en 1659 y fue reactivada por ME en lisados que contenán pHMB. La mayor parte de la actividad de CP en lisados HM1 se encontró en una fracción que sedimentó a 15,600×g en tanto que predominó en los sobrenadantes de lisados 1659. El aumento de la virulencia de *E. histolytica* correlacionó por tanto con un aumento notable de la enterotoxicidad *in vitro* y la actividad de dos CPs (53 y 75 kDa), sugiriendo que estas proteinasas son importantes factores de patogenicidad (Publicado en *Experimental Parasitology* 1995;80).

Inmunidad Intestinal Antiamibiana

Los cambios observados en el transporte iónico del intestino en respuesta a la infección por el nemátodo parásito *Trichinella spiralis* han generado la mayor parte de la información disponible sobre las vías mediante la cuales las señales antigénicas se traducen en una respuesta local del epitelio intestinal. La influencia de la reacciones inmunológicas sobre el transporte de líquidos y electrolitos se expresa dramáticamente después de la estimulación antigénica. En la triquinosis murina la secreción de líquido por el estímulo antigénico ocurre hasta varios días después de la infección primaria, pero en cambio se evoca al cabo de sólo unos minutos en la infección secundaria. En preparaciones de intestino aislado de ratas infectadas con *T. spiralis* el reto antigénico induce secreción neta de Cl^-.

En la amibiasis ha sido demostrada la inmunidad local del intestino a través de la detección de coproanticuerpos, de células productoras de anticuerpos en las placas de Peyer y de anticuerpos de clase IgA antiamibianos en la bilis de ratas inmunizadas intracecalmente, así como también en la leche y el calostro de humanos. Nosostros encontramos que la inmunización de ratas por vía intragástrica con trofozoitos de *E. histolytica* fijados con glutaraldehido incrementa la producción de células formadoras de anticuerpos antiamibianos en las placas de Peyer y el bazo. La lámina propia del ciego de ratas inmunizadas mostró una infiltración de eosinófilos que contenían anticuerpos homocitotrópicos de la clase IgE. El reto antigénico (con lisado amibiano)

a preparaciones de ciego montadas en cámaras de Ussing provenientes de ratas que habían sido inmunizadas con amibas fijadas, provocó un incremento de Icc y DP. Estos hallazgos sugieren que hay hipersensibilidad de tipo I en el ciego de ratas inmunizadas oralmente, y que los eosinófilos y la IgE pueden ser factores importantes en la inmunidad local y por tanto en la patogénesis de la amibiasis intestinal. Los antígenos amibianos o las amibas mismas podrían reaccionar con anticuerpos IgE homocitotrópicos de producción local y así provocar degranulación de células cebadas y basófilos intestinales y liberación local de sustancias farmacológicamente activas. Por otra parte, estos anticuerpos podrían jugar también un papel protector regulando la inflamación temprana que acompaña a la amibiasis intestinal invasora con la participación de eosinofilos, células cebadas y cristales de Charcot-Leyden (Manuscrito enviado a publicación).

Nuestro modelo experimental nos ha permitido detectar las lesiones morfológicas y electrofisiológicas más tempranas (vacuolización subnuclear y caída inmediata de DP, Icc y R) descritas hasta ahora en la amibiasis experimental. Tiene además las ventajas que ya hemos mencionado sobre otros modelos *in vitro* y en animales enteros. Otra contribución interesante de nuestro trabajo es que la comparación de las propiedades de los trofozoítos de virulencia aumentada con sus progenitores atenuados parece ser una estrategia más adecuada que la usada habitualmente, contraria a la que aquí usamos nosotros, que consiste en comparar la virulencia de variantes amibianas atenuadas —seleccionadas a priori por su fenotipo deficiente en funciones celulares que podrían no tener relación directa con la virulencia— con la de las cepas parentales de las que proviene.

Como los factores determinantes del curso de la infección amibiana son la invasividad del parásito y la inmunidad del hospedero, actualmente estamos realizando —en colaboración con la doctora Miriam Pedroso, inmunóloga del Centro Nacional de Sanidad Agropecuaria de La Habana— experimentos electrofisiológicos en los que desafiamos con lisados amibianos a preparaciones de intestino de animales inmunizados con trofozoítos solos o acompañados con la toxina del cólera (el mejor adyuvante conocido por vía oral) y un inmunoestimulante (ß-1,3 glucano, derivado de la pared de levaduras), producido en Cuba. Estos experimentos son paralelos a otros que estamos llevando a cabo para caracterizar la inmunidad antiamibiana intestinal y

sistémica, tanto en su rama inductora (formación de células productoras de anticuerpos en las placas de Peyer y el bazo) como en la efectora (anticuerpos en el suero, en el contenido intestinal y en las heces, así como anticuerpos, interleucinas y linfocitos T y B en la lámina propia del intestino grueso).

Finalmente, vale la pena recordar que la prevención de muchas infecciones de las mucosas podrá realizarse de manera más conveniente con vacunas orales polivalentes, las vacunas del futuro. El desarrollo de estas vacunas depende de avances en el conocimiento de los componentes y mecanismos de la fisiología e inmunidad de las mucosas, en el conocimiento de los factores y mecanismos de la virulencia de los patógenos adherentes y la identificación y clonación de los genes correspondientes y, por último, de la producción de los antígenos relevantes con técnicas de DNA recombinante o mediante síntesis química. Como las vacunas orales contra las enfermedades infecciosas de los países pobres no interesan a los países desarrollados, nosotros estamos obligados a desarrollarlas.

CAPITULO I

Introducción

ANTECEDENTES GENERALES

Patógenos adherentes y vacunas

Aunque las enfermedades infecciosas causan un tercio de las muertes en todo el mundo, su impacto es aún mayor en los países subdesarrollados, donde son 30 veces más frecuentes (Commission on Health Research for Development, 1990). Las infecciones respiratorias agudas y las enfermedades diarréicas son las enfermedades infecciosas de mayor importancia global: a ellas se deben tres cuartas partes de las muertes por infecciones en el mundo (Commission on Health Research for Development, 1990) y en México ocupan los primeros lugares como causas de muerte, sobre todo en niños (Martínez-Palomo y Sepúlveda, 1989). Estas enfermedades se deben a la colonización y daño de las mucosas —las cubiertas epiteliales húmedas que delimitan y recubren el interior de los aparatos respiratorio, digestivo y genitourinario, cuya superficie total es unas 200 veces mayor que la superficie de la piel— por agentes microbianos llamados patógenos adherentes (McGhee y Kiyono, 1993).

La patogenicidad —capacidad de producir enfermedad— puede deberse a la invasividad o a la toxigenicidad de los agentes infecciosos (Henderson, 1989). La invasividad es la capacidad de destruir localmente los tejidos colonizados, en tanto que la toxigenicidad es la capacidad de producir daño en sitios relativamente distantes. Aunque no hay patógenos invasores o toxigénicos puros, algunas especies se distinguen por el predominio de uno de los dos mecanismos (Henderson, 1989).

Los efectos de los microorganismos toxigénicos —entre los que sobresalen los que causan la tosferina, la difteria, el tétanos y el botulismo— pueden explicarse por los efectos a distancia de una toxina, especie molecular homogénea codificada y sintetizada por el agente patógeno (Plotkin, 1993). Desde el siglo pasado se sabe que la vacunación con toxinas inactivadas ("toxoides") es muy eficaz para inmunizar contra los efectos de la infección por los organismos toxigénicos (Henderson, 1989).

La mayoría de los agentes patógenos no son toxigénicos sino invasores, y sus efectos globales son determinados por múltiples factores (Henderson, 1989). Esto significa que su virulencia —grado de la enfermedad que pueden provocar— resulta de la suma de los efectos

debidos a todos los factores patogénicos que expresan durante su interacción con los animales infectados. El desarrollo de vacunas efectivas contra los patógenos invasores es más difícil que contra los toxigénicos porque requiere del conocimiento de la patogenicidad y la producción y utilización de diversos antígenos —moléculas reconocidas como extrañas por el animal y potencialmente capaces de evocar inmunidad protectora— relevantes, capaces de inducir inmunidad protectora y duradera (McGhee y Kiyono, 1993).

La vacunación es uno de los medios más eficaces y baratos para prevenir las enfermedades infecciosas. Sin embargo, el desarrollo de nuevas vacunas que ayuden a prevenir las enfermedades de mayor incidencia en los países pobres está prácticamente paralizado porque el mercado de fármacos nuevos es mucho más lucrativo que el de las vacunas (Henderson, 1989).

Por otra parte, desde hace buen tiempo se sabe que la vacunación oral contra los patógenos adherentes es más simple e induce inmunidad protectora más intensa y duradera que la vacunación parenteral (McGhee y Kiyono, 1993). Sorprende que entre las vacunas más empleadas en todo el mundo contra enfermedades infecciosas prevenibles de esta manera, sólo dos —las del cólera y la polio— se administren por vía oral (The Merck manual of diagnosis and therapy, 1987). Por ello resulta tan importante desarrollar nuevas vacunas orales efectivas como mejorar las parenterales que ya existen.

El desarrollo de vacunas orales eficaces contra los patógenos adherentes depende de avances en el conocimiento de dos áreas clave: 1) la inmunidad de las mucosas —las barreras epiteliales que constituyen la primera y más extensa e importante línea de defensa contra los patógenos adherentes—, y 2) los mecanismos patogénicos de los agentes infecciosos, particularmente la identificación y caracterización de los factores moleculares que provocan las alteraciones estructurales y metabólicas en las enfermedades infecciosas y pudiesen servir como antígenos en las vacunas correspondientes.

Para comprender los conceptos que siguen, conviene tener una idea de la anatomía del intestino. El tubo digestivo está formado por varias capas; la más interna es la mucosa, y determina la "luz" o espacio donde se encuentra el contenido intestinal; la más externa es la serosa, capa lisa y brillante formada por el peritoneo (Aguirre-Cruz y López-Revilla, 1990). La mucosa está formada por una sola capa de células epiteliales. Por debajo de ella se encuentra la

lámina propia, con buen número de células maduras —"efectoras"— del sistema inmune de las mucosas, que se encargan de la defensa contra las infecciones causadas por los agentes que llegan por la vía oral (Vega-López et al. 1993). Bajo la lámina propia se encuentra la capa submucosa y envolviendo a ésta se halla la capa muscular, formada por músculo liso responsable del peristaltismo —movimiento involuntario del intestino que impulsa el bolo alimenticio (Aguirre-Cruz y López-Revilla, 1990).

Las mucosas, primera línea de defensa contra las infecciones

La respuesta inmune de las mucosas está sujeta a mecanismos únicos, especialmente en cuanto a las funciones de reconocimiento y efectoras. A ella no se aplican ciertos dogmas de la respuesta inmune periférica, especialmente los concernientes al manejo de los antígenos (Panja et al. 1993).

Varios grupos han demostrado que las células epiteliales intestinales (CEI) tienen un papel activo en el manejo de los antígenos, ya que pueden expresar antígenos de histocompatibilidad de clase II, que son moléculas de la superficie celular involucradas en la presentación de antígenos, las cuales eran consideradas como exclusivas de las células presentadoras de antígenos "profesionales" (Lycke y Svennerhom, 1990). Por otra parte, al entrar en contacto con los patógenos adherentes en la mucosa, las CEI secretan interleucina 8, mediador quimiotáctico que atrae a otras células del sistema inmune al sitio de contacto (Eckmann et al. 193).

Cuando los patógenos adherentes colonizan la mucosa intestinal, sus antígenos son capturados y procesados por los órganos linfoides —acúmulos de linfocitos y de otras células del sistema inmune— localizados inmediatamente por debajo de las mucosas, que en el intestino son llamados "placas de Peyer".

Los antígenos inducen una respuesta inmunológica en las placas de Peyer, caracterizada por la diferenciación y proliferación de linfocitos especializados, con actividad citotóxica o capacidad de producir anticuerpos contra los antígenos de los agentes infecciosos que los inducen (McGhee y Kiyono, 1993). Entre los linfocitos productores de anticuerpos predominan los que sintetizan inmunoglobulinas de la clase A (IgA), que son los anticuerpos más abundantes en el organismo y los que predominan en las secreciones de las mucosas y en el contenido intestinal

(Biewenga et al. 1993).

Los linfocitos estimulados por los antígenos que son captados y procesados en las placas de Peyer y en los órganos linfoides correspondientes de la mucosa respiratoria, genitourinaria y otras (Sminia et al. 1990), emigran primero a través de los vasos y los ganglios linfáticos, luego pasan a la circulación general, y ya maduros se establecen en la lámina propia —capa subyacente a la mucosa— en las diferentes regiones de los aparatos digestivo, respiratorio y genitourinario, y en las glándulas salivales, lacrimales, etc. (McGhee y Kiyono, 1993).

En los últimos años ha quedado claro que la inmunidad local de las mucosas inducida por la infección previa con un patógeno adherente protege mejor y permite respuestas inmunes más rápidas y eficientes contra infecciones subsecuentes por la misma especie de patógeno que la inmunidad sistémica inducida por vacunación parenteral.

Propiedades electrofisiológicas de las mucosas

Las preparaciones de mucosa han sido usadas para estudiar el transporte de electrolitos en los diferentes segmentos del intestino de mamíferos.

Cuando un segmento de mucosa intestinal es montado en una cámara de Ussing —aparato formado por dos hemicámaras simétricas— en las que es bañado inicialmente con soluciones idénticas en ambas superficies, la solución que baña la cara externa (serosa) puede volverse eléctricamente positiva respecto a la cara interna, luminal o mucosa (Ussing y Zerahn, 1951). A la diferencia de cargas eléctricas entre la solución que baña la cara externa (serosa) y la que baña la interna (luminal o mucosa) de las preparaciones —generada por cambios en la concentración de iones, debidos al transporte selectivo de los mismos a través de la mucosa— se le llama diferencia de potencial transepitelial (DP). La inyección de corriente en dirección opuesta despolariza el tejido; la corriente necesaria para anular ("fijar") la DP es llamada corriente de corto circuito (Icc) y se puede aplicar automáticamente mediante un aparato llamado "fijador de voltaje". La resistencia eléctrica del tejido (R) se calcula con la ley de Ohm, como el cociente DP/Icc (volts/amperes); la conductancia total del tejido (G) es la inversa de R (Navarro-García et al. 1994).

Los efectos de sustancias que afectan la función e integridad de los epitelios en general y de la mucosa intestinal en particular pueden ser analizados determinando las propiedades electrofisiológicas de preparaciones montadas en cámaras de Ussing (Fasano et al. 1990. McGowan et al. 1983, Navarro-García et al. 1992). La presencia de parásitos en el intestino provoca un estrés que induce cambios locales en la estructura y función de los tejidos (Hinterleitner y Powell, 1973). Como las respuestas fisiológicas hacia los parásitos intestinales están condicionadas por el estado inmune del animal infectado, el comprender cómo las señales antigénicas se traducen en cambios fisiológicos podría permitirnos prevenir o regular las respuestas inmunopatológicas y estimular las respuestas favorables (Castro, 1989).

ANTECEDENTES DIRECTOS

Amibiasis intestinal

Las amibas —formas vegetativas o trofozoítos— del protozoario parásito llamado *Entamoeba histolytica* colonizan y dañan la mucosa del intestino grueso, causando la amibiasis intestinal (Espinosa-Cantellano y Martínez-Palomo, 1991). De ahí las amibas pueden diseminarse a otros órganos y provocar la amibiasis extraintestinal (Pérez-Tamayo, 1986). A pesar de la gravedad de los casos de amibiasis extraintestinal, la amibiasis intestinal es la manifestación clínica más frecuente de enfermedad, la fuente de transmisión de la infección y el blanco natural para la vacunación (Navarro-García et al. 1994).

Las amibas histolíticas son fundamentalmente invasoras, porque las lesiones que causan consisten en zonas de necrosis en los sitios colonizados por los parásitos (Takeuchi y Phillips, 1975, Pratrap y Gilman, 1970). La gran dificultad que reviste el trabajo en la amibiasis intestinal experimental ha sido el factor determinante para que se investigue preferentemente la amibiasis extraintestinal.

Los dos grupos de mecanismos antagónicos cuyo balance determina que se produzca o no la enfermedad son la invasividad de cada cepa amibiana y la inmunidad local contra las amibas.

Los modelos experimentales de amibiasis intestinal y extraintestinal pretenden reproducir las lesiones amibiásicas en roedores mediante inoculación directa de amibas en el ciego —porción inicial del intestino grueso, donde se inicia la amibiasis intestinal— y del hígado —órgano más comúnmente invadido por amibas a partir del intestino—, respectivamente. El modelo más común de amibiasis intestinal consiste en inducir lesiones en el intestino grueso de cobayos mediante inoculación de amibas en el ciego (Takeuchi y Phillips, 1975; Anaya-Velázquez et al. 1985); este procedimiento es laborioso, poco reproducible, y no se sabe si induce inmunidad local. El modelo más común de amibiasis extraintestinal consiste en producir abscesos en el hígado de hámster mediante inoculación directa de los parásitos en el órgano; la infección es reproducible y parece inducir inmunidad duradera (Tsutsumi et al. 1992).

En la amibiasis intestinal las células epiteliales de la mucosa del intestino grueso deben jugar un papel importante, ya que cuando ocurre la infección por quistes de *E. histolytica*, éstos se transforman en trofozoítos y se adhieren a dichas células de la mucosa para iniciar la invasión. Los eventos iniciales de la interacción de los trofozoítos con la mucosa del intestino grueso probablemente determinan que ocurra o no la amibiasis intestinal invasora.

Mecanismos de invasión de E. histolytica

Para determinar el mecanismo de invasión de la mucosa por las amibas se han obtenido datos de tres fuentes: 1) biopsias de humanos, 2) amibiasis experimental, 3) interacción amibas-células in vitro.

Prathap y Gilman (1970) clasificaron en cuatro tipos las alteraciones de biopsias de pacientes con amibiasis intestinal aguda: 1) lesión inespecífica, que consiste en engrosamiento de la mucosa; 2) lesión inicial, o depresión de la superficie mucosa con pérdida de mucina y deformación de las células epiteliales de columnares a cuboidales; 3) pequeñas lesiones en el espacio interglandular del epitelio, a través del cual ocurre la invasión de los trofozoítos; y 4) "úlcera en botón" que abarca la capa mucosa y la muscularis mucosae.

Takeuchi y Phillips (1975) inocularon cobayos intracecalmente con amibas y encontraron que cuando los trofozoítos están cercanos a las células epiteliales, las microvellosidades se acortan, se hacen irregulares y algunas veces desaparecen. Cuando se produce el contacto con las

amibas, el citoplasma apical de las células epiteliales se proyecta hacia las amibas y las células dañadas se despegan de las células vecinas. Una observación que estos autores comparten con otros es que la invasión amibiana se inicia en la superficie interglandular del epitelio.

Knight et al. (1974) empleando monocapas de células en cultivo observaron que las lesiones detectables con el microscopio electrónico se inician cuando los trofozoítos establecen contacto con las células. En los estadios iniciales de la interacción no detectaron daño en la membrana plasmática, sino más bien en algunos componentes citoplásmicos, especialmente las mitocondrias, las cuales mostraron vacuolización y pérdida de las crestas. En interacciones prolongadas observaron daños focales en la membrana plasmática.

En los trabajos mencionados en los que se emplearon biopsias y células in vitro, los autores proponen que la citólisis y la destrucción de los tejidos son debidas a enzimas liberadas por las amibas

Recientemente mediante el uso de jerbos (*Meriones unguiculatus*), modelo animal de gran utilidad porque en ellos se puede producir experimentalmente tanto amibiasis intestinal como hepática, hemos encontrado que cuando se inoculan trofozoítos vivos por vía intracecal se observa un incremento en la producción de moco durante las primeras seis horas. Entre las 24 y 72 h postinoculación aparecen lesiones microulcerativas en la mucosa. Infiltrado inflamatorio y edema en la lámina propia estuvo asociado a focos superficiales de necrosis. A las 96 h, la mucosa cecal tuvo una aspecto casi normal y no fueron detectadas amibas vivas. Estos resultados nos permitieron sugerir al jerbo como un modelo experimental para el análisis de los estados tempranos de la amibiasis intestinal invasora (M. Shibayama, F. Navarro-García, R. López-Revilla, A. Martínez-Palomo y V. Tsutsumi, enviado a pulicación)

Modelos in vitro *de amibiasis intestinal*

Las preparaciones de intestino en cámaras de Ussing ha sido de gran utilidad para estudiar los mecanismos de acción de las enterotoxinas y los mecanismos patogénicos e inmunológicos en enfermedades entéricas causadas por bacterias, protozoarios y nemátodos.

McGowan y otros encontraron en 1983 que los lisados de trofozoítos de *E. histolytica* afectan el transporte electrolítico cuando son añadidos al lado seroso de preparaciones de mucosa

de colon de rata o íleon de conejo; los cambios inducidos fueron similares a los causados por varias substancias neurohumorales, lo cual se explicó cuando el mismo grupo encontraró que los lisados amibianos contienen serotonina, neurotensina y sustancia P inmunoreactiva (McGowan y Donowitz, 1988). Sin embargo, estos efectos fueron detectados solamente con lisados añadidos a la cara serosa pero no a la cara luminal de las preparaciones, que es el sitio por donde los trofozoítos invaden naturalmente el intestino grueso.

Para estudiar los efectos electrofisiológicos de los factores patogénicos de *E. histolytica* y los posibles cambios en la sensibilidad a estos factores que pudieran ser inducidos por la inmunidad local contra la amibiasis intestinal, nosotros decidimos tratar de ensayar los efectos de las amibas o sus productos sobre preparaciones de intestino grueso con todas sus capas —provenientes de animales normales o inmunizados con amibas o antígenos derivados de ellas. Los experimentos electrofisiológicos que confirmaron el transporte activo de sodio por el epitelio intestinal fueron realizados con preparaciones de mucosa intestinal desprendida de segmentos de pared intestinal en los que se eliminaron las capas muscular y serosa.

En mi tesis de maestría describí los efectos iniciales de lisados de *E. histolytica* sobre la fisiología (propiedades eléctricas dependientes del transporte de sodio) y la morfología (daño estructural al microscopio óptico) del colon de conejo. En nuestro modelo experimental montamos en cámaras de Ussing a segmentos de colon que incluían todas las capas del intestino, desde la mucosa hasta la serosa, los cuales tratamos con lisados de trofozoítos colocados en la cara mucosa. Encontramos que los lisados de *E. histolytica* HM1 causaron una caída inmediata en la diferencia de potencial (DP), la corriente de corto circuito (Icc) y la resistencia eléctrica (R) de las preparaciones. La velocidad de caída por lisados de 50,000 trofozoítos fue similar a la observada en preparaciones hipóxicas (mantenidas en cámaras de Ussing con Ringer sin burbujeo de la mezcla con 95% O_2-5% CO_2). Los efectos electrofisiólogicos causados por los lisados y por la hipoxia correlacionaron con el daño morfológico observados con microscopía óptica. Los lisados amibianos parecieron causar: i) formación de pequeñas vacuolas entre la capa epitelial y la membrana basal que originan la separación de la capa epitelial de la lámina propia; ii) edema entre la capa epitelial y la lámina propia que permite una mayor separación entre las mismas; iv) pérdida de la integridad de las células epiteliales interglandulares; v) separación de las células

epiteliales de la lámina basal (80 min de interacción) que en sus inicios (aprox 60 min) provocan una caída en la R (Navarro-García et al. 1992, 1993).

Instrumentación para estudiar la electrofisiología de la mucosa intestinal

Como la mucosa intestinal tiene valores de DP e Icc mucho menores que los de la piel y la vejiga de rana (Schultz et al. 1981) —para los cuales se diseñaron las primeras cámaras de Ussing y los fijadores de voltaje correspondientes— (Schultz et al. 1977), el estudio de sus propiedades electrofisiológicas exige equipo electrónico más sensible. Por ello una parte significativa de nuestro proyecto implicó el diseño, la manufactura, la prueba y la mejoría del equipo de registro.

Con la ayuda del doctor José Luis Reyes y la colaboración de especialistas en diversas disciplinas nos iniciamos en la electrofisiología de los epitelios de transporte y hemos llegado a conjuntar un instrumental confiable para la caracterización de las propiedades de preparaciones de intestino en cámaras de Ussing. Este equipo está formado por tres tipos de componentes, los cuales han sido diseñados y producidos en el CINVESTAV y la Universidad Autónoma de San Luis Potosí (en la cual constituyeron dos tesis de licenciatura de sendos estudiantes de física de la Facultad de Ciencias): 1) cámaras de Ussing, 2) fijadores de voltaje, 3) sistema automático de captura y manejo de datos mediante una computadora personal.

Las cámaras y las celdas, de plástico acrílico, fueron elaboradas en el taller del CINVESTAV. Los fijadores de voltaje fueron diseñados y manufacturados por David Elías (Sección de Bioelectrónica, Departamento de Ingeniería Eléctrica del CINVESTAV). El sistema automatizado de captura, registro y manejo de los datos consta de dos tarjetas (una como interface en el fijador de voltaje; otra como interface en la computadora para capturar los registros electrofisiológicos) y un programa de cómputo con 1) un menú para anotar los datos generales de cada experimento y seleccionar los intervalos apropiados para la fijación automática del voltaje y 2) el registro de los resultados electrofisiológicos en forma tabular y su transformación para ser exportados a un programa graficador.

Inmunidad local en la amibiasis intestinal

La invasión tisular de las amibas a la mucosa puede iniciar la producción de anticuerpos circulantes (Trissl 1982). Es posible, por otra parte, que el establecimiento de las respuestas inmunes locales no requieran de la penetración tisular (Grundy et al. 1983). Cerca del 80% de los pacientes con disentería amibiana presentan coproanticuerpos, en tanto que menos del 4% de los pacientes con infecciones parasitarias no amibianas y sólo el 2% de los testigos sanos tuvieron estos anticuerpos (Sharma et al. 1981). La naturaleza de estos anticuerpos no ha sido establecida con claridad. Sin embargo, existen estudios que permiten suponer que al menos parte de ellos pertenecen a la clase IgA (Shaalan y Baker, 1970).

En otros estudios de coproanticuerpos en amibiasis se encontró que sólo el 55% de los casos presentaron anticuerpos tres semanas después del diagnóstico, cuando hacían su aparición los anticuerpos circulantes (Martínez-Cairo et al. 1979). Esta observación ilustra el curso de la respuesta inmune en la amibiasis intestinal invasora y resalta lo efímero de la respuesta inmune secretora contra *E. histolytica* (Grundy et al. 1983). Además se ha demostrado la presencia de anticuerpos de la clase IgA contra amibas en la bilis de ratas inmunizadas intracecalmente (Acosta et al. 1983), así como en la leche (Grundy et al. 1983) y en el calostro humano (Acosta et al. 1985).

Todos los tipos de inmunoglobulinas pueden encontrarse en las secreciones, aunque sólo la IgA y la IgE son secretadas con preferencia (Tomasi y Bienenstock, 1968). En nuestro laboratorio hemos detectado títulos significativos de IgA secretoria (SIgA) por Dot-ELISA en siete de 42 muestras de calostro que provenían de madres de niños recien nacidos con diarrea (López-Revilla et al. 1991). La IgA intestinal sirve como una barrera contra la colonización y la penetración de microorganismos patógenos (Bazin, 976) e inhibe la absorción intestinal de antígenos (Stokes et al 1975). Es posible suponer que los anticuerpos secretores juegan un papel similar en el caso de *E. histolytica* (Trissl, 1982). Además, recientemente se ha reportado incremento en la producción de células formadoras de anticuerpos antiamibianos en placas de Peyer y bazo de ratones inmunizados con amibas fijadas con glutaraldehido por vía intragastrica, intraperitoneal e intrarrectal, lo que indica que se puede inducir (sitios inductores placas de Peyer y bazo) una respuesta inmune local contra los trofozoítos de *E. histolytica* (Moreno-Fierros et

al. 1994). Estos anticuerpos pueden ser detectados en líquido intestinal y suero (Moreno-Fierros et al. 1995), los cuales reconocen diferentes antígenos según la vía de inmunización (Moreno-Fierros, comunicación personal).

La inmunidad secretora, por efímera que fuese, podría desempeñar un papel más crítico que la inmunidad sistémica en el mantenimiento del balance entre la amibiasis estrictamente luminal y la amibiasis intestinal invasora y contribuir así a la gran diferencia que existe entre la prevalencia de la infección amibiana y su morbilidad (Walsh y Warren, 1979).

La producción local de anticuerpos anti-*E. histolytica* en el intestino podría contribuir a la patogenia de la amibiasis (Kretschmer, 1984). Los antígenos amibianos o las propias amibas podrían reaccionar con anticuerpos IgE homocitotrópicos de producción local y desencadenar así la desgranulación de células cebadas y basófilos intestinales (Miller, 1980) y con ello promover la liberación de varias sustancias farmacológicamente activas. Estas a su vez podrían dañar al huesped al abrir canales intercelulares y así dar acceso hacia estructuras más profundas en la mucosa, o bien perturbar las respuestas inmunes celulares locales (Plaut et al. 1975). Por otra parte, estos anticuerpos también podrían jugar un papel protector al regular la inflamación temprana que acompaña a la amibiasis intestinal invasora, en la que participan eosinófilos, cristales de Charcot-Leyden y células cebadas (Juniper et al 1958). Este último hallazgo da crédito a la existencia de reacciones anafilácticas locales en la amibiasis (Kagan, 1973). Tal vez parte de la sintomatología de la disentería amibiana aguda (dolor, cólico, tensión abdominal, tenesmo y heces sanguinolentas) se debe a un fenómeno de anafilaxia local (Barth et al 1966) y no tanto, como se ha propuesto, a la serotonina y la neurotensina presentes en *E. histolytica* (McGowan et al. 1983, 1984). Finalmente, podría ocurrir una sutil interrelación entre anticuerpos IgA bloqueadores y los anticuerpos IgE anafilácticos en las secreciones, ambos resultantes de una exposición intestinal mínima a *E. histolytica*, en la modulación de los estadios tempranos de la interacción huésped-parásito (Kretschmer, 1989).

El proceso de penetración intestinal de las amibas o de difusión de antígenos amibianos subcelulares, que juegan un papel tan importante en el establecimiento de respuestas humorales locales, podría tener un papel similar en el establecimiento de respuesta inmunes celulares locales contra *E. histolytica* (Ganguly y Waldman, 1978). La incidencia relativamente alta de reacciones

de hipersensibilidad retardada documentada en sujetos sanos (20%) y en pacientes con rectocolitis amibiana (47.1%) en áreas en las que la amibiasis es endémica (Kretschmer et al. 1972) sugiere que existe, igualmente, inmunidad celular local.

No sería sorprendente encontrar evidencia histológica de reacciones de hipersensibilidad retardada si se aplicara antígeno amibiano en la submucosa del intestino de animales previamente inmunizados (Kretschmer, 1989). Después de todo, la hipersensibilidad retardada en la piel no es sino la focalización del elemento circulante de la inmunidad celular (Turk, 1980), aunque, como en la inmunidad mucosa humoral, existe cierta independencia entre la versión local (mucosa) y la sistémica de la inmunidad celular (Bogger-Goren et al. 1984). Sin embargo, la información sobre inmunidad celular mucosa contra *E. histolytica* es virtualmente nula (Trissl, 1982). Los exudados generados en el caso de amibiasis intestinal contienen linfocitos que son liberados a la luz intestinal (Pittman et al. 1973) y se ha sugerido que se trata de células T supresoras y citotóxicas (Ferguson et al. 1983a). En presencia de anticuerpos específicos, estos elementos linfoides podrían ser cruciales en la defensa contra infecciones entéricas (Mkwananzi et al. 1976; Lowell et al. 1980) a través de reacciones de citotoxicidad celular dependiente de anticuerpos, incluyendo una versión mediada por anticuerpos de clase IgA (Tagliabue et al. 1984). Alternativamente, estas células podrían tener una función opuesta funcionando con predominancia como células supresoras o tolerantigénicas (Ferguson et al. 1983b).

Estudios usando cinematografía muestran que el suero inmune *in vitro* o la gamaglobulina antiamibiana pueden matar un 90% de trofozoítos de *E. histolytica* en 60 min (Chévez et al. 1973). Estos efectos son termoestables y pueden ser eliminados preincubando el suero o la fracción de inmunoglobulinas con células completas u homogenados a 4°C. Por otro lado, la adhesión celular mediada por una lectina amibiana (Meza et al. 1987) así como la glicoproteína citotóxica (Kobiler et al. 1981) pueden ser inhibidas por anticuerpos IgG. También se ha reportado la inhibición de los procesos de adhesión por anticuerpos monoclonales anti-*E. histolytica* de la clase IgA en celulas MDCK y HT-29 (Leyva et al 1992), así como por anticuerpos monoclonales dirigidos contra el antígeno de superficie de 66 kDa (Vohra, et al. 1992). La actividad proteolítica de células completas o lisados amibianos puede también ser inhibido por anticuerpos antiamibianos. Además, la inyección de suero inmune de humanos

previene parcialmente la formación de abscesos hepáticos en hámsteres inoculados con amibas virulentas (Sepúlveda et al. 1974).

La ausencia de infección en los lactantes de madres infectadas con *E. histolytica* ha sido interpretada como protección pasiva devida a IgA secretoria antiamibiana en la leche de estas madres (Islam et al. 1988). Sin embargo, esta observaciones pueden también ser debidas al efecto deletéreo que la leche humana de sujetos no inmunes tiene sobre *E. histolytica* (Sepúlveda y Martínez-Palomo 1984).

A pesar de que se ha demostrado el efecto deletéreo que pueden tener *in vitro* los anticuerpos humorales sobre el parásito, no se sabe cúal es el papel que estos anticuerpos puedan tener *in vivo*, ya que se han hecho experimentos aislados de inmunización humoral pasiva.

Es posible que la IgA secretoria y quizás la IgE puedan ser verdaderos factores protectores en la amibiasis, aunque probablemente den una corta protección temporal contra la amibiasis invasora.

Inmunofisiología de las mucosas

Hace poco más de una década Castro (1982) propuso que la función epitelial es controlada en parte por elementos de la lámina propia, a lo cual llamó regulación inmunológica o "inmunofisiología" de los epitelios. A partir de entonces el grupo de Castro ha mostrado que las reacciones inmunológicas en varias infecciones intestinales con helmintos, en especial con *Trichinella spiralis*, afectan la diferenciación y desarrollo de las células epiteliales así como las actividades de secreción, absorción y digestión (Castro et al. 1979, Hessel et al. 1982, Harari y Castro 1988).

Por otro lado, se ha reportado la participación directa de elementos inmunes en cambios estructurales y en el transporte electrolítico por células epiteliales. Russell (1986) describió la participación de las células cebadas en la regulación del transporte electrolítico en el intestino de cuyos infectados con *T. spiralis*. Este tipo de respuesta en el epitelio está involucrado en la reacciones anafilácticas producidas por *T. spiralis*, y que causan alteraciones en el transporte de iones (Castro et al. 1987). Este estado fisiológico alterado en respuesta a mediadores derivados

de células cebadas hace que el intestino funcione como un tejido efector en el proceso de expulsión del parásito y dentro de este proceso se ha encontrado la participación de la secreción de cloro inducida por el antígeno (Harari et al. 1987). También se ha encontrado que la atrofia de las vellosidades intestinales, la hiperplasia de las criptas y la expulsión de *T. spiralis* en el intestino delgado del ratón son mediados por células T (Manson-Smith et al. 1979).

PLANTEAMIENTO DEL PROBLEMA

Nuestro interés está centrado en la compresión de los dos grupos de mecanismos antagónicos que participan en la amibiasis intestinal y cuyo balance de determinar que se produzca o no la enfermedad: i) la invasividad de cada cepa amibiana y ii) la inmunidad local contra las amibas.

Nuestro modelo de amibiasis intestinal *in vitro* (desarrollado durante mi trabajo de tesis de maestría) detecta daños electrofisiológicos (funcionales) y morfológicos (estructurales) agudos a la mucosa intestinal, por lo cual resultaba de interés extender este trabajo para caracterizar los daños a la mucosa y comparar los efectos de cepas de alta virulencia con los de cepa de baja virulencia, asi como caracterizar el mecanismo diferencial entre ambas cepas. Por otro lado como el daño causado a las células epiteliales por los lisados amibianos, observado en nuestro modelo, pudiera ser afectado por la respuesta inmunológica local del intestino, nos parecía muy importante investigar si la inmunización de animales experimentales modificaba los efectos de los lisados *E. histolytica* sobre las preparaciones intestinales. En caso de observar cambios, caracterizaría el mecanismo inmunológico.

Por ello en el curso de este proyecto analizamos: (a) El efecto de la dosis de trofozoítos lisados de *E. histolytica* y el tiempo de exposición a los lisados sobre la electrofisiología (DP, Icc y R) y la morfología (microscopía óptica y electrónica) de preparaciones de intestino grueso. (b) La comparación de una cepa de alta virulencia con su progenitora de baja virulencia, así como la posible participación de las proteasas en la virulencia amibiana. (c) Determinar si la respuesta inmune local previene o modifica el daño provocado por los lisados amibianos en preparaciones de intestino de animales previamente inmunizados y caracterizar el mecanismo inmune que pudiera estar participando en este modelo.

Los resultados experimentales se presentan en los siguientes tres capítulos que corresponden a cada uno de los aspectos abordados durante el trabajo.

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CAPITULO II

Electrophysiology and Immunity of the Mucosa in Intestinal Infections

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Electrophysiology and Immunity of the Mucosa in Intestinal Infections

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Abstract
We present here some of the major concepts and approaches to study the electrophysiology of the intestinal mucosa, and review the pathophysiology of intestinal infections caused by enteropathogenic bacteria, protozoa - especially our own work on experimental amebiasis using intestinal preparations mounted in the Ussing chamber - and nematodes, and finally discuss briefly the immunophysiology of the intestinal mucosa. (*Arch Med Res* 1994; 25:253)
KEY WORDS: Intestinal mucosa; Ussing chamber; Intestinal infections; Vaccines; Enteropathogenic bacteria; Protozoan parasites; Nematode parasites; Mucosal immunity; Mucosal immunophysiology.

Introduction

Although infections cause one third of deaths in the world and thus as a group represent a major global health problem, their impact is tremendous in developing countries, where they are about 30 times more frequent as causes of death than in industrialized countries (Table 1) (1). Acute respiratory infections and diarrheal diseases are initiated at the corresponding mucosal surfaces, cause at least three fourths of the global deaths by infections (Table 2) (1), and are also among the major causes of death in Mexico (Table 3) (2).

Advances in the knowledge of the components and mechanisms of mucosal physiology and immunity have led, during the last decade, to the conception of the mucosal or secretory immune system (3), and of a new interdisciplinary field called "mucosal immunophysiology" (4). Coupled to the identification of pathogenic factors of specific mucosal infectious agents and the ability to clone the corresponding genes and produce them *in vivo* via recombinant DNA for use as antigens, these conceptual advances suggest promising approaches in the development of new vaccines against mucosal infections which may have the convenience of being administered by the oral route. In a retrospective look, it is surprising that although prevention of most mucosal infections might be performed in a more convenient and effective manner through oral vaccination, most of the current vaccines are administered by the parenteral route (Table 4) (5).

In this paper we have attempted to present the concepts and approaches we feel are crucial in the research on the local immunity of mucosal infections. We review recent studies on the pathogenesis and immunity of intestinal infections mainly through electrophysiologic approaches, and especially our own results on experimental amebiasis, using intestinal preparations mounted in Ussing chambers. We limit ourselves to the intestine because (i) the interrelations of different compartments of the secretory immune system suggest that orally administered vaccines may be effective against intestinal infections as well as against infections of other mucosae, (ii) the experimental

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Table 1
Estimated Number of Deaths by Cause in Industrialized and Developing Countries, 1985 (thousands of persons)

Cause	World	Industrialized countries	Developing countries
Infections	17,006 (35%)	506 (1%)	16,500 (34%)
Circulatory, degenerative	12,430 (25%)	5,930 (12%)	6,500 (13%)
Rest of causes	19,509 (40%)	4,608 (9%)	14,901 (30%)
All Causes	48,945 (100%)	11,045 (23%)	37,896 (87%)

Adapted from "Health research: essential link to equity in development", Oxford, 1990.

Table 2
Estimated Number of Deaths by Infections in Industrialized and Developing Countries, 1985 (thousands of persons)

Cause	World	Industrialized countries		Developing countries	
Acute respiratory infections	7,768	368	2.2%	7,400	44%
Diarrheal diseases	5,025	25	0.1%	5,000	29%
Tuberculosis	2,840	40	0.2%	2,800	16%
Malaria	1,000	-	-	1,000	6%
Others	373	73	0.4%	300	2%
Total	17,006	506	2.9%	16,500	97%

Adapted from "Health research: essential link to equity in development", Oxford, 1990.

manipulation of the intestine is simpler than that of other organs, and finally, (iii) our experience is limited to intestinal infection models.

Electrophysiology of the Intestinal Mucosa

Stripped mucosal preparations have been used to study electrolyte transport in various segments of the intestine (6-8). In the mammalian large intestine, active Na^+ absorption occurs through the cell surface, and secretion such as that of Cl^- occurs through cells of the crypts (9-11). When an epithelium is mounted between two Ussing-type hemichambers (Figure 1), and bathed with identical solutions on both surfaces, the serosal solution is electrically positive with respect to the mucosal solution (12). These differences in electric charge are the basis of electrophysiological properties. The difference of electric charge between the serosal and the mucosal side of the epithelial layer, due to the difference in ion concentrations, is called transepithelial potential difference (PD). The passage of current in opposite direction depolarizes the tissue. With enough depolarizing current the difference of voltage between the two sides can be reduced to zero. Under these conditions the preparation is in a short-circuit state and the current necesary to clamp the voltage of the preparation is called short-circuit current (Isc) (7). Electrical resistance (R) represents the relation of PD/Isc, whereas the total tissue conductance (G) is 1/R (13). The intestinal mucosa resembles other sodium transporting epithelia, such as amphibian skin and urinary bladder, because they have PD and Isc that normally result from active Na^+ transport from the mucosa to the serosa (14,15). Since the electrical properties of mucosal preparations depend mainly on sodium transport (14), their functionality may be checked with specific inhibitors such as amiloride (7) and oaubain (cf. 15,16). The addition of amiloride - a drug that blocks sodium entry on the apical plasma membrane - to the mucosal solution results in an Isc fall to zero, while addition to the serosal solution has no effect (14). Preparations challenged with ouabain (Figure 2), inhibitor of the Na^+/K^+ ATPase located in the basolateral plasma membrane of epithelial cells, suffer a significant decrease in their Isc and PD values, whereas their R values are nearly constant (16).

Pathophysiology of Intestinal Infections

Immediate effects of various substances affecting the function and integrity of epithelia in general and of the intestinal mucosa in particular may be determined by monitoring the electrophysiologic properties of epithelial

Table 3
Five Main Causes of Death in Mexico, 1984[a]

Cause	Deaths	Rate per 100,000
Heart diseases	51,328	67.2
Accidents	46,242	60.6
Malignant neoplasms	31,885	41.8
Intestinal infections	29,806	39.1
Influenza and pneumonia	27,249	35.7

[a]Martínez-Palomo & Sepúlveda, 1989.

Table 4
Routes of Immunization Against 18 Specific Infections, 1987

Infection	Route
Cholera	O/P[a]
Diphtheria	P
Hemophilus influenzae b	P
Hepatitis B	P
Influenza	P
Measles	P
Meningococcal meningitis	P
Mumps	P
Pertussis	P
Pneumococcal pneumonia	P
Poliomyelitis	O/P
Rubella	P
Tetanus	P
Tuberculosis	P
Typhoid fever	P
Rabies	P
Plague	P
Yellow fever	P

[a]O = oral; P = parenteral.

preparations mounted in Ussing chambers (13,17-19). This approach is now being increasingly used to understand the mechanisms of action of enterotoxins and the pathogenic and immunologic mechanisms of enteric diseases caused by bacteria (13,20-22), protozoa (16,17,19,23,24) and nematodes (25-27), as will be seen below.

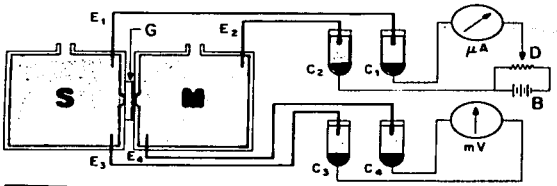

Figure 1. Diagram of gut preparations mounted in a Ussing-type chamber. B, Battery; C, calomel cells; D, potential divider; E, electrodes; G, gut fragment with its mucosa facing the right hemichamber (M) and its serosa facing the left hemichamber (S). (From Reference 23, with permission.)

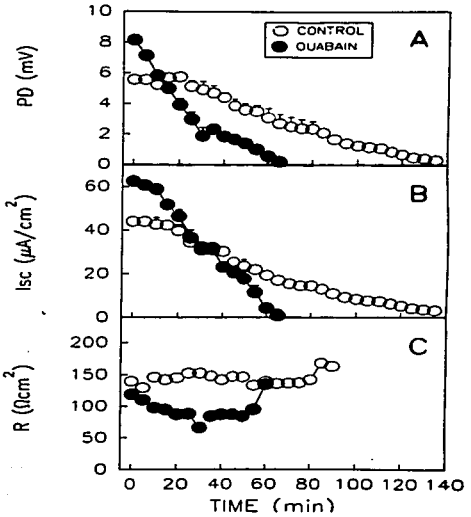

Figure 2. Effects of ouabain on rabbit colon preprarations. Ouabain was added to both hemichambers to a final concentration of 10^{-4} M. The symbols represent PD, Isc and R values (mean ± standard error) at each time point (n = 5). (From Reference 16, with permission.)

Enteropathogenic Bacteria

Most intestinal bacteria that affect ionic secretion or absorption are related to diarrhea production. Although the adenylate cyclase-cAMP system was the first mediator identified in electrolyte secretion, little is known about how this system is implicated in the etiology of *in vivo* intestinal secretion, such as that provoked by cholera

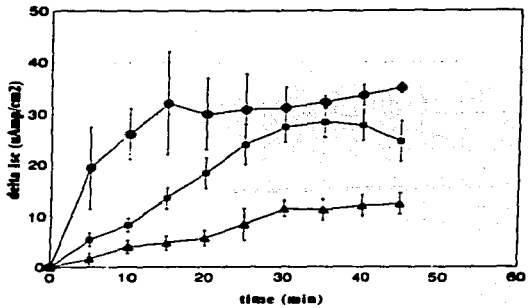

Figure 3. Effects of *Escherichia coli* heat-stable enterotoxin (ST) and of enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) on the Isc of rabbit ileal mucosa. ST at 10^{-7} M (■); EAST1 at 10^{-4} and 10^{-7} M (●); phosphate buffered saline (▲). The symbols represent mean ± standard error of Isc at each time point (n = 4). (From Reference 34, with permission.)

toxin, prostaglandins and *Escherichia coli* heat labile enterotoxin which is usually related with increased mucosal adenylate cyclase activity (20).

Vibrio cholerae produces the copious diarrhea characteristic of cholera by means of a potent enterotoxin, cholera toxin (CT). Purified CT added to the mucosal side of human ileum preparations mounted in Ussing chambers cause a net Na^+ movement of 0.6 μEq/cm²·h and significant Cl^- net secretion, whereas normal ileum preparations have a Na^+ net movement of 2.6 μEq/cm²·h (21). This effect is similar to that obtained when theophylline (inhibitor of cyclic nucleotide phosphodiesterase) is added. However, attenuated *V. cholerae* vaccine strains mutated in the genes encoding CT are still capable of causing mild to moderate diarrhea. When these CT-negative strains were examined in Ussing chambers, a second toxin was found that increases the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junction, or *zonula occludens*. Production of this factor (named ZOT, for *zonula occludens* toxin) correlates with diarrheagenicity in volunteers (22). The effect of *E. coli* heat-stable enterotoxin (ST) on intestinal alkaline secretion and transepithelial PD has been reported in denervated segments of rat jejunum, ileum, and proximal colon. ST causes an increase of alkaline secretion in the jejunum but not in the ileum or colon. The jejunal effect of ST may be due to a stimulation of bicarbonate secretion and/or an inhibition of Na^+/H^+ exchange. ST causes a rise in colonic PD, whereas only a small response is found in the jejunum and no effect in the ileum. The effects of lidocaine on PD and alkaline secretion are similar to those of hexamethonium; they diminish the effect of ST on PD and cause a small inhibition of the ST-induced alkaline secretion. These findings suggest that ST exerts, via the enteric nerves, a profound influence on the jejunal transport mechanisms responsible for the changes in PD, whereas the influence on alkaline secretion seems not to be mediated via enteric nerves. Thus, the extent of the enteric nervous control of epithelial function differs for different transport functions (28).

Several categories of *E. coli* are firmly established as important enteropathogens (29), including enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC) and the more recent diarrheagenic category, enteroaggregative *E. coli* (EAggEC) associated with persistent diarrhea in children (30,31). EIEC strains are a significant cause of diarrheal diseases and dysentery in young children. Supernatants and cell lysates of two EIEC strains tested in rabbit ileal loops stimulate moderate fluid accumulation without tissue damage; secretory activity has been confirmed in Ussing chambers, where these strains significantly increase Isc without altering tissue conductance (13). In general, choleragen and heat-stable enterotoxin variants of *E. coli* STa (ETEC) cause an increase in the Isc of ileal mucosa by stimulation of anioic net secretory flux (32,33).

Enterotoxic activity in culture filtrates of EAggEC strains has been studied in rabbit ileal mucosa mounted in Ussing chambers. Filtrates from strain 17-2 cause the greatest rises in PD and Isc (Figure 3) which are different from those caused by ST (34). These effects have been attributed to a low-molecular-weight, partially heat-stable, plasmid-encoded enterotoxin, named EAST1 (for EAggEC heat-stable enterotoxin 1). EAST1 is genetically and immunologically distinct from ETEC STa (34). Recently, Savarino et al. (35) identified clones expressing the gene encoding EAST1, employing Ussing chambers to detect the effects on rabbit ileal mucosa (35).

Protozoa

Amebiasis. Intestinal amebiasis is the most common form of amebic disease and the source for its transmission. The balance between the virulence of infecting *Entamoeba histolytica* trophozoites and local immunity presumably determines the degree of invasion of the intestinal mucosa by the parasite. However, current animal models of intestinal amebiasis have the disadvantages of being quite laborious, having low reproducibility, and producing lesions which are detectable only several days after intracecal inoculation with the parasite (36-38). Several potentially pathogenic factors of amebae have been identified (39-41), besides proteinases with cytotoxic activity (42-45), a pore-forming protein (46,47), and substances similar to certain neurohormones (17,48).

To study the short-time cytopathic effects of amebae on epithelial cells, Orozco et al. (49) used electron microscopy and recorded transepithelial electric resis-

tance (R) of cultured epithelial cells in contact with live trophozoites and extracts. Electrophysiologic records of R detected damage in the cellular monolayers after 2 min of interaction. Damage was progressive with time and maximum after 60 min. EM analysis showed the passage of a molecular tracer between epithelial cells. These results suggest that the initial functional damage of cell monolayers is manifested by the opening of tight junctions.

McGowan and Donowitz (50) have analyzed the role of secretagogues in the pathophysiology of the diarrhea due to *E. histolytica* infection. They found that *E. histolytica* lysates added to the serosal side of rabbit ileum or rat colon affect electrolyte transport in a manner similar to several neurohumoral substances (17). The same group found that amebal lysates contain serotonin, neurotensin, and immunoreactive substance P, and that lysate-induced effects are partially inhibited by antibody to serotonin (50). More recently this group reported that the effect of lysates on ion transport has both calcium-dependent and calcium-independent components and demonstrated that arachidonic acid metabolites of the cyclooxygenase pathway, not dependent on calcium entry, are probably involved in the *E. histolytica*-induced changes in colonic transport (51). They also showed that amebic lysates do not contain prostaglandin E_2 (PGE_2) but stimulate PGE_2 release from rat colonic mucosa and increase colonic cAMP, and that such effects are partially inhibited by PGE_2, indomethacin, piroxicam and mepacrine (51). These results suggest that neurohormones present in *E. histolytica* trophozoites may act directly on colonic tissue to stimulate intestinal secretion, probably via a calcium-dependent mechanism blockable by verapamil, or indirectly via stimulation of PGE_2 generation and release from the rat colon through a cAMP-dependent mechanism; the effects appear to be separated, with cAMP-dependent secretion predominating in this model of colonic amebic diarrhea (51). However, the effects are elicited only when lysates are added to the submucosal side, not when they are added to the luminal side of preparations, where trophozoites naturally invade the large intestine. In contrast, we have found that treatment with *E. histolytica* HM1 trophozoite lysates on the luminal side of colon preparations kept in basal culture medium for amebae cause an immediate decrease of PD and Isc, whereas lysates derived from the same number of *Entamoeba invadens* PZ trophozoites (strain virulent in reptiles) have no effect (23).

Lysed *E. histolytica* HM1 trophozoites added to the luminal side of rabbit colon preparations kept either in basal culture medium for amebae (23) or in Ringer's solution (19) induce an immediate fall of PD, Isc and R, whereas the same dose of *E. invadens* PZ trophozoite lysates has undetectable electrophysiologic effects. The earliest microscopic sign of damage in intestinal segments exposed to *E. histolytica* lysates is the presence of

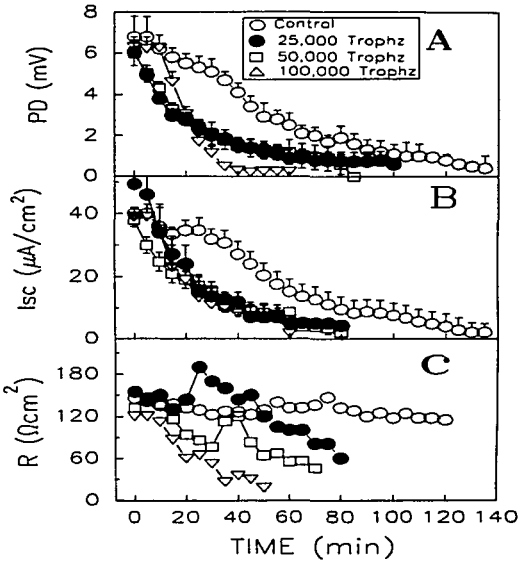

Figure 4. Effects of variable doses of *E. histolytica* HM1 trophozoite lysates on rabbit colon preparations. Lysed trophozoites (2.5×10^4, 5×10^4 or 1×10^5) were added to the mucosal hemichamber. The symbols represent the values (mean ± standard error) at each time point (n = 5). (A) PD, (B) Isc, and (C) R. (From Reference 24, with permission.)

translucent vacuoles located at the bases of epithelial cells, later followed by detachment of the epithelial layer from the lamina propria and destruction of interglandular epithelial cells (16). The magnitude of the electrophysiologic effects, mainly on PD and R, depends on the dose of amebal lysates (Figure 4) and correlates with the degree of the microscopic lesions observed. The smallest dose (25,000 lysed trophozoites) induced the formation of small translucent vacuoles which under the light microscope are located between the epithelial layer and the lamina propria; with the electron microscope such vacuoles are located under the nuclei, at the bases of epithelial cells. With the intermediate dose (50,000 lysed trophozoites), the epithelial layer started to separate from the lamina propria, and incipient necrosis, mostly of interglandular epithelial cells, also occurred; under the electron microscope these preparations had shortened epithelial cells devoid of intercellular junctions, with deformed nuclei and fragmented microvilli. The highest dose (100,000 lysed trophozoites) caused massive

desquamation and destruction of interglandular epithelial cells (24). In preparations treated with the intermediate dose of lysates, after 15 min of exposure there was incipient vacuolization at the bases of epithelial cells. After 40 min, the vacuoles had increased in size and intercellular junctions were lost. After 80 min, when PD and Isc had just reached nil values, there were large subnuclear vacuoles at the bases of the interglandular epithelial cells, the loss of tight junctions was complete and there was also a massive loss of interglandular epithelial cells (24).

The magnitude of the electrophysiologic effects, mainly on PD and R, depended on the dose and correlated with the type and degree of the microscopic lesions observed (16). The beginning of microvilli fragmentation coincided with the initiation of PD and Isc decay, properties known to depend mainly on the Na^+ influx by microvilli pumps and channels (14). The formation of cytoplasmic vacuoles in the basal portion of the epithelial cells may be associated with the water transport blockage known to accompany Na^+ transport inhibition (52). The loss of intercellular junctions, coinciding with the beginning of R decay, may be due to the free passage of ions through the paracellular route.

The structural lesions just described are similar to those already seen in human and experimental intestinal amebiasis. For instance, in human biopsies Prathap and Gilman (53) found mucosal edema, epithelial cell shortening and interglandular lesions, whereas in guinea pigs Takeuchi and Phillips (36) observed shortening and disappearance of microvilli, loss of tight junctions, and interglandular epithelial cell damage. On the other hand, Knight et al. (54) showed that during the early interaction of amebae with RK13 monolayer cells there was no ultrastructural damage of the plasma membrane, but the cytoplasm contained empty vacuoles and mitochondrial crests were lost. We have been able to detect the earliest electrophysiologic and morphologic lesions described up to now in experimental intestinal amebiasis: PD and Isc decay, and vacuolation at the bases of epithelial cells. Our model also has the following advantages over other *in vivo* and animal models: a) it is closer to intestinal amebiasis because the target is a full-thickness intestinal segment and not cultured cells, b) the electrophysiologic properties of the colon segments exposed to trophozoite lysates may be correlated with their morphologic and biochemical changes, c) immediate location of the microscopic lesions is possible because the intestine-lysate interaction takes place in a specified minimal area, d) the results are reproducible, in contrast with those of animals inoculated intracecally, which are inconsistent and take several days to develop.

The gerbil is being increasingly considered as an appropriate animal model of intestinal amebiasis, characterized by microulcerative mucosal lesions, inflammatory infiltrate, edema of the lamina propria associated with necrotic foci, as well as increased cecal mucus production (55-57). We have recently found that *E. histolytica* trophozoite lysates also increase the rate of decay of the values of electrophysiologic properties of gerbil cecum preparations mounted in Ussing chambers, and a clear correlation between the *in vivo* virulence of *E. histolytica* trophozoites for gerbil intestine with the *in vitro* virulence of amebal lysates for gerbil cecum preparations in Ussing chambers. A low virulence *E. histolytica* HM1 substrain that has been axenically cultured for 15 years was compared with another substrain that had just been passagged through hamster liver to make it recover its virulence. The more virulent substrain produced much more tissue damage when inoculated in the large intestine of gerbils, and a much faster decay of the electrophysiologic properties in Ussing-chambered gerbil cecum, and its overall cysteine proteinases activity was 25 times higher than that of the less virulent substrain (F. Navarro-García, L. Chávez-Dueñas, V. Tsutsumi, R. López-Revilla, manuscript in preparation).

Giardiasis. *Giardia lamblia* is a common cause of diarrhea worldwide and is the most frequently recognized etiologic agent of waterborne disease in North America (58,59). Despite the prevalence of this pathogen, the pathophysiology of *Giardia* infections is not fully understood. Clinical malabsorption in giardiasis has been associated with severe villus atrophy and crypt hyperplasia in some cases (60-62), and with normal histology in others (63-65).

With Ussing chambers Buret et al. (66) found that the infection in gerbils resulted in decreased jejunal glucose-stimulated electrolyte, water, and 3-O- methyl-D-glucose absorption (shown by Na^+ and Cl^- transport, PD and Isc), whereas the ileum was similar in infected and control animals. Infection resulted also in crypt hyperplasia associated with an increased enterocyte migration rate. Villus height decreased in the duodenum, was unchanged in the jejunum, and again increased in the ileum of infected animals. Epithelial microvilli were markedly shortened, and brush border surface area decreased in the jejunum and ileum of infected animals. Thymidine kinase activity increased in enterocyte villi isolated from the duodenum but did not change in the jejunum and ileum. Thus, the infection causes electrolyte, solute, and fluid malabsorption associated with decreased jejunal brush border surface area, and the diarrhea associated with giardiasis is therefore caused by malabsorption rather than active secretion (66).

Ciguatera. Ciguatera fish poisoning is a clinical syndrome consisting of a combination of gastrointestinal (diarrhea, vomiting, abdominal pain) and neurological symptoms occurring after eating toxin-containing fish. The toxin (or combination of toxins) responsible for ciguatera is produced by the benthic dinoflagellate

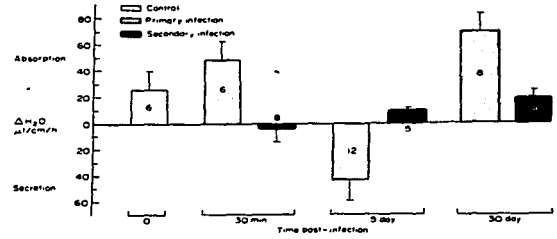

Figure 5. Net intestinal water movement as a function of time in rats uninfected and primarily or secondarily infected with *Trichinella spiralis*. Number of animals in each group is shown within the bars. (From Reference 25, with permission.)

Gambierdiscus toxicus. Electrophysiologic studies in amphibian skin suggest that ciguatoxin and ciguatoxin-like toxins have a direct effect on excitable membranes involving modifications in Na^+ permeability (67) and competitive inhibition of the membrane polarizing action of Ca^{++} (67,68). In order to define the pathophysiologic mechanisms responsible for the diarrheal component of the illness, Fasano et al. (69) examined the effect of crude and fractionated toxin preparation on isolated rabbit ileal tissue in a Ussing chamber (69). Both toxin preparations gave a striking increase in transepithelial PD and Isc. Enterotoxic activity seemed to be mediated by calcium. When examined by light microscopy the intestinal mucosa was not damaged, suggesting that the toxins involved in ciguatera fish poisoning directly stimulate intestinal fluid secretion without accompanying tissue damage, and that calcium is the second messenger mediating the process.

Nematodes

Ionic transport changes in the small intestine found in response to the nematode parasite *Trichinella spiralis* have provided most of the available information about the pathways through which antigenic signals are transduced into gut epithelial responses (18).

The influence of immunological reaction on electrolyte and fluid transport is dramatically expressed after antigenic stimulation. In murine trichinosis, fluid secretion occurs several days after primary infection (Figure 5), but within minutes of secondary infection (25). *In vivo* changes in ionic transport, representing correlates of fluid secretion, are detectable within seconds of antigenic stimulation. Net Cl^- secretion is induced by antigenic challenge of isolated intestine from *T. spiralis*-infected rats (27).

Net ion transport by the jejunum of rats immunized against *T. spiralis* on challenge with parasite-derived antigen, measured in Ussing chambers, rapidly expresses a biphasic rise and fall in Isc. Antigenic challenge of sensitized jejunum causes the release of 5-hydroxytryptamine, histamine and PGE_2, known to affect sodium transport (26).

Immunophysiology of the Intestinal Mucosa

Three major systems control water and electrolyte transport by the intestinal epithelium (70). The enteric nervous system is the best studied of these systems; through its different components - extrinsic sympathetic and parasympathetic nerves, enteroendocrine cells, and, most importantly, the submucosal and myenteric plexuses - it constitutes the "little brain" that controls most gastrointestinal functions. Hormones of the endocrine system, like gastrin and secretin, are important in regulating pancreatic and gastric secretion; a classical example of endocrine control of intestinal electrolyte transport is the renin-aldosterone axis, which serves to increase sodium and water absorption by the large intestine. The regulatory system more recently recognized is the immune system, which is capable of complex regulatory control of transport through the secretion of various soluble mediators that alter ion and water movement both directly and indirectly via the enteric nervous system.

At least two general types of immune system-mediated alterations in intestinal ion transport have been identified: those mediated by degranulation of mast cells, and those initiated by phagocytes. There are also transport alterations initiated by T lymphocytes (Figure 6). An important and useful way to define the action of immune cell mediators is to add these substances directly to the serosal bath of Ussing-chambered intestine preparations (70).

Castro (4) proposed more than 10 years ago that epithelial functions are in part regulated by elements of the lamina propria, which he called immunological regulation or "immunophysiology" of the epithelia. Since then, Castro and co-workers have shown that immunological reactions in several intestinal helminthic infections, especially by *T. spiralis*, affect epithelial cell differentiation and development, as well as secretory, absorptive, and digestive activities (25,71,72). Studies of intestinal nematode infections have shown that elements of the lamina propria contribute to the control of epithelial structure and function, through their immunological capabilities. Experimental evidence shows that non-epithelial cells influence directly, indirectly, or in both ways, the morphology of intestinal villi and the epithelial structure, differentiation, secretion, and absorption (4).

Direct participation of immune elements in structural and electrolyte transport changes of epithelial cells has been reported. Russell (73) described the participation of mast cells in electrolyte transport regulation in *T. spiralis*-

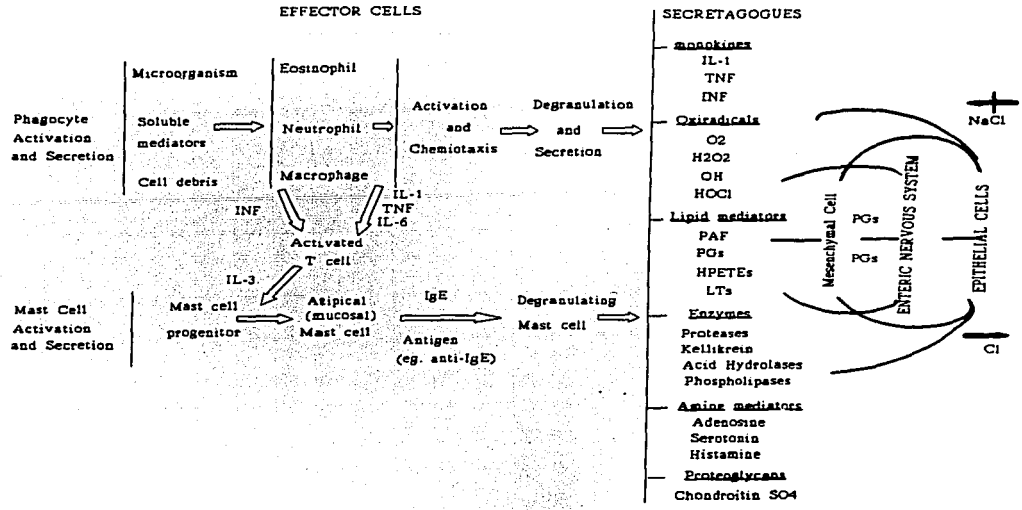

Figure 6. Control of intestinal electrolyte transport by the immune system. At the effector level there is activation of phagocytes and mucosal mast cells, secretion of preformed products and newly synthesized inflammatory mediators. At the amplification level, there are mesenchymal cells such as endothelial cells and fibroblasts responding to the mediators released by leukocytes that then act as paracrine cells that release neurotransmitters. Inflammatory mediators may act either on these intermediate targets, or directly on mucosal epithelia. When acting as final targets, epithelial cells respond with increased chloride secretion and decreased NaCl absorption. (Modified from Reference 70.)

infected guinea pig intestine. This type of epithelial response is involved in the anaphylatic reaction produced by *T. spiralis* that causes alterations of the ion transport (26). This altered physiologic state in response to the mast cell-derived mediators makes the intestine to function as an effector tissue in the process of parasite expulsion into which the participation of antigen-induced Cl^- secretion has been found (74). In rat small intestine it has been found that villous atrophy, crypt cell hyperplasia and rejection of *T. spiralis* are mediated by T cells (75).

The parasites cause anatomical and fuctional changes in the smooth muscle layers of the gut wall. In the small intestine of rat infected with *Nippostrongylus brasiliensis* there is a marked thickening of the muscularis externa, due to hypertrophy of the longitudinal smooth muscle layer. Analogous observations have been reported in rats and guinea pigs infected with *T. spiralis*, rats with *Moniliformis dubius*, horses with *Parascaris equorum* and pigs with *Ascaris suum*. The causes of increased muscle mass in these infections are unknown but may involve physical effects of parasites, parasite-derived substances, or chemical mediators of inflammation (18).

Observations on the immune regulation of smooth muscle function implicate the activity of the enteric nervous system in some of the pathways through which antigens induce effector responses. Challenge of the myenteric plexus from *T. spiralis*-infected guinea pigs with parasite-derived antigen leads to changes in transmembrane potential and electrical resistance in the impaled neurons. The transduction of the antigenic signal to elicit the neuronal changes presumably involves a response initiated by mast cells, suggesting that enteric neurons in sensitized gut tissue respond physiologically to antigenic stimulation (18).

In amebiasis the participation of local immunity has been suggested through coproantibodies (76), antibody producing cells in Peyer's patches (77) and IgA antiamebic antibodies in the bile of intracecally immunized rats (78), as well as in human milk (79) and colostrum (80,81).

Local production of anti-*E. histolytica* IgE antibodies in the intestine may contribute to the pathogenesis of amebiasis (82). Amebic antigens or amebae may react with homocytotropic IgE antibodies of local production and thus cause degranulation of intestinal mast cells and basophils and release of several pharmacologically active

substances (83). On the other hand, these antibodies may also play a protective role to regulate the early inflammation that accompanies intestinal invasive amebiasis, with participation of eosinophils, mast cells and Charcot-Leyden crystals (84).

Using electrophysiology as a tool we are currently performing experiments to determine the participation of local immunity in intestinal amebiasis; these experiments consist of *in vitro* challenging intestinal preparations obtained from animals previously immunized with trophozoites administered by the intragastric, intracecal and intraperitoneal routes. The rate of fall of the electrophysiologic properties in Ussing-chambered preparations challenged *in vitro* with amebal lysates seems to depend on the route of immunization. Another interesting preliminary finding is that in intestinal preparations of immune rats challenged with amebal lysates, there is a rapid initial increase of Isc and PD, followed by a rapid fall of both values and the resulting loss of electrical resistance; these effects suggest that there is type I hypersensitivity in intestinal amebiasis, as predicted by Kretschmer (85).

Models of the mast cell-initiated intestinal secretion can be created through the addition of anti-IgE immunoglobulin to the serosal solution of Ussing-chambered rat colon (86). The various roles of the different classes of inflammatory mediators or of the enteric nervous system can be dissected by pretreating the tissue with specific antagonists (e.g., antihistamines) or mediator synthesis inhibitors (e.g., cyclooxygenase blockers) in the Ussing chamber prior to stimulation, or by the addition of neurotransmitter inhibitors (70).

Castro (18) proposed not long ago that it may be advantageous or even necessary for researchers studying parasites of the gastrointestinal tract to view the host response from the immunophysiological perspective in order to reconcile phenomena or events that cannot be readily explained by traditional disciplinary approaches or concepts. In a relatively short time this interdisciplinary approach has become essential, as we have tried to show in this review.

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CAPITULO III

Dose- and Time-Dependent Functional and Structural Damage to the Colon Mucosa by *Entamoeba histolytica*

Parasitol Res (1993) 79:517–522

Parasitology Research

Dose- and time-dependent functional and structural damage to the colon mucosa by *Entamoeba histolytica* trophozoite lysates

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Abstract. Analysis of the initial interaction between *Entamoeba histolytica* trophozoites and the large intestine is impossible in humans and very difficult in experimental animals. To circumvent this obstacle we treated the luminal side of full-thickness rabbit colon segments mounted in Ussing-type chambers with trophozoite lysates of the *E. histolytica* HM1 virulent strain. Exposure to lysates for up to 90 min produced dose- and time-dependent effects on the colon, consisting of (a) increased decay rates for potential difference, short-circuit current, and transmural resistance and (b) mucosal damage ranging from vacuolation at the bases and shortening of epithelial cells to the loss of intercellular junctions, destruction of microvilli, and necrosis of interglandular epithelial zones. This acute model of intestinal amebiasis is sensitive, fast, and reliable.

Amebiasis, infection by *Entamoeba histolytica* trophoites, is the third global cause of death by parasitism (Walsh 1986). Virulent *E. histolytica* trophozoites invade the mucosa of the large intestine and from there they may colonize and damage extraintestinal organs (Pérez-Tamayo 1986). Early intestinal invasion events include the adhesion of trophozoites to epithelial cells, the lysis and dislocation of such cells, the degradation of the basement membrane, and the penetration of amebae to the lamina propria (Prathap and Gilman 1970; Takeuchi and Phillips 1975).

Although the initial interaction of trophozoites with the colon mucosa probably is crucial for invasion, an analysis of this process is impossible in humans and extremely difficult in experimental animal models due to their variability and to the nearly impossible task of localizing the earliest microscopic lesions produced by intracecal inoculation of amebae (Ghadirian and Konshvan 1984; Anaya-Velázquez et al. 1985).

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An in vitro model of acute intestinal amebiasis developed recently in our laboratory (López-Revilla et al. 1992; Navarro-García et al. 1992) allowed us to detect and characterize the earliest electrophysiologic and morphologic intestinal lesions caused by *E. histolytica* trophozoite lysates. In this paper we describe such lesions and correlate them with the dose and time of exposure to amebal lysates.

Materials and methods

Colon preparations

Segments measuring 2–3 cm in length were obtained from the ascending colon of New Zealand (1.5–2 kg) male rabbits under sodium pentobarbital anesthesia. The excised portions were placed in ice-cold Ringer's solution for mammals at 4° C and gassed with an O_2/CO_2 (95%/5%) mixture. Each intestinal segment was cut open along its mesenteric border, rinsed clean of luminal contents and extended, and kept in gassed Ringer's solution (López-Revilla et al. 1992). Full-thickness rabbit colon segments were mounted between the circular openings (diameter, 6 mm) of two adjacent Ussing hemichambers. Each hemichamber was filled with 2.5 ml of gassed Ringer's solution and kept at 37° C during bubbling of only the serosal side. Transmural potential difference (PD) and short-circuit current (Isc) values were recorded at 5-min intervals by means of a voltage clamp as described elsewhere (López-Revilla et al. 1992).

Treatment with amebal lysates

Entamoeba histolytica trophozoites of the HM1 virulent strain (López-Revilla and Gómez-Domínguez 1988) were obtained from log-phase axenic cultures and washed by centrifugation in TYI basal culture medium (Diamond et al. 1978). Washed pellets were stored under liquid nitrogen (Navarro-García et al. 1992). At the beginning of each experiment, the pellets were diluted with Ringer's solution to obtain suspensions containing 100000 trophozoites/ml; amebal lysates were obtained through a freeze-thawing cycle and then mixed with appropriate volumes of the same diluent at 37° C. Aliquots (2.5 ml) of suspensions containing 25000, 50000, or 100000 lysed trophozoites were added to the mucosal hemichamber of preparations that had been mounted for 5 min; fresh Ringer's

solution at 37° C was then added to the serosal hemichamber and gassed with O_2/CO_2.

By the method of Bradford (1976), we determined an average content of 245 μg protein/10⁶ trophozoites of the *E. histolytica* HM1 strain using bovine serum albumin as the standard. Fresh lysates that were added to the luminal hemichamber had the typical protein profile observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Feingold et al. 1985) and did not modify the pH or the osmolarity of the Ringer's solution.

Electron microscopy

At the end of each electrophysiology experiment, the preparations were removed from the Ussing chambers and immediately fixed for 1 h with 2.5% glutaraldehyde dissolved in 0.1 *M* sodium cacodylate (pH 7.4). Fixed segments were cut into 1-mm-thick sections, fixed for a further 2 h, postfixed with osmium tetroxide, dehydrated with ethanol at increasing concentrations, and finally embedded in Epon 812. Semithin (1-μm-thick) sections were stained with toluidine blue to be examined with a light microscope. Thin (60- to 90-nm-thick) sections were stained with uranyl acetate followed by lead citrate and examined with a Zeiss EM-10 electron microscope.

Statistical analysis

Electrophysiologic properties were compared using Student's *t* test on data subjected to square root or arcsin transformation (Navarro-Garcia et al. 1992). Initial decay rates were calculated by regression of the PD and Isc curves resulting from each treatment.

Results

Electrophysiologic effects of treatment with variable doses of lysates

Control preparations. The mean initial PD value (PD_o = 6 mV) was stable for 30 min and then fell gradually to reach nil within about 120 min at a decay rate of −0.004 mV/min (Fig. 1). The mean initial Isc value ($Isc_o = 32\ \mu A/cm^2$) was stable for about 35 min and then fell to reach nil within about 125 min at a decay rate ($-0.003\ \mu A\ cm^{-2}\ min^{-1}$) proportional to that of PD. In contrast, the mean initial value for transmural resistance ($R_o = 185\ \Omega cm^2$) was stable for at least the 2 *h* during which the electrical activity was monitored (Fig. 1).

Treated preparations. When 50000 lysed trophozoites were added to the luminal side of preparations, we confirmed that they produce an immediate decrease in PD and Isc (López-Revilla et al. 1992; Navarro-García et al. 1992). PD decay was associated with the decay of R (Fig. 1) as previously found (Navarro-García et al. 1992).

In view of the results obtained using a single dose, we analyzed the effects of a smaller (25000), an identical (50000), and a larger (100000) dose of lysed trophozoites. Preparations treated with the highest dose lost their PD faster (Fig. 1A), with the initial decay rate being −0.033mV/min, some 8 times higher than and significantly different ($P < 0.05$) from the "spontaneous" decay rate of −0.004mV/min (Table 1). On the other hand, preparations treated with 25000 and 50000 lysed trophozoites had PD decay rates of about −0.015 mV/min that were nearly identical and did not significantly differ ($P = 0.63$, Table 1).

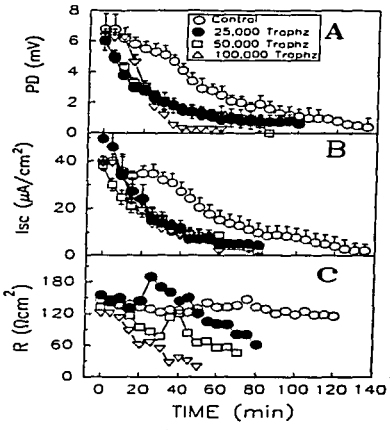

Fig. 1A–C. Effect of variable doses of *Entamoeba histolytica* HM1 trophozoite lysates on the **A** PD, **B** Isc, and **C** R values obtained for full-thickness rabbit colon preparations. Both hemichambers contained Ringer's solution gassed with O_2/CO_2; 5 min after each preparation had been mounted, 50000 lysed trophozoites (*Trophz*) were added to the luminal hemichamber and gassing was sustained only in the serosal hemichamber. Initial values (mean ± SE) were: $PD_o = 6 \pm 2$ mV and $Isc_o = 35 \pm 12\ \mu A/cm^2$. R values were obtained from PD/Isc ratios at each time point; only reliable R values (derived from Isc values higher than $2\ \mu A/cm^2$) are plotted. R_o (mean ± SE) was $140 \pm 20\ \Omega cm^2$. The symbols represent values obtained at each time point ($n = 5$)

Table 1. Deterioration of the electrical properties of rabbit colon preparations treated with various doses of *Entamoeba histolytica* HM1 trophozoite lysates

Dose[a]	Stability[b] (min)	Decay time[c] (min)	Decay rates (min^{-1})		
			PD[d] (mV)	Isc[d] ($\mu A/cm^2$)	R (Ωcm^2)
0	35	130	−0.004	−0.003	−0.155
25000	5	80	−0.015	−0.014	−1.130
50000	5	80	−0.015	−0.019	−1.282
100000	5	55	−0.033	−0.020	−2.238

[a] Number of lysed trophozoites added to each preparation
[b] Time during which the initial values ($DP_o = 6 \pm 2$ mV; $Isc_o = 35 \pm 12\ \mu A/cm^2$; $R_o = 140 \pm 20\ \Omega cm^2$) were stable
[c] Time at which PD and Isc reached nil values
[d] Obtained by linear regression of decay curves obtained during the first 10 min of treatment

The Isc decay rates were -0.014, -0.019, and -0.020 $\mu A\ cm^{-2}\ min^{-1}$ for preparations treated with 25000, 50000, and 100000 lysed trophozoites, respectively (Fig. 1B). These rates differed significantly ($P < 0.05$) from those observed in untreated preparations, whose average value was -0.003 $\mu A\ cm^{-2}\ min^{-1}$ (Table 1).

In relation to R, the decay rate of untreated preparations was -0.155 $\Omega cm^2/min$, whereas the values determined for preparations treated with 25000, 50000, and 100000 lysed trophozoites were -1.13, -1.28, and -2.24 $\Omega cm^2/min$, respectively (Fig. 1C, Table 1). The rates of R decay in preparations exposed to 50000 and 100000 lysed trophozoites differed significantly ($P < 0.05$) from the value determined for untreated preparations, which did not differ from the decay rate observed in preparations exposed to 25000 lysed trophozoites ($P = 0.87$).

Morphologic effects of treatment with variable doses of lysates

Light microscopy. Semithin sections of the preparations used in the electrophysiology experiments showed dose-dependent lesions. Even after having been mounted for 120 min (Fig. 2A), untreated samples appeared normal in semithin sections. Treatment with 25000 lysed trophozoites for up to 80 min caused slight damage consisting of small translucent vacuoles located between the epithelial layer and the lamina propria (Fig. 2B). Samples treated for 80 min with 50000 lysed trophozoites showed increased desquamation of interglandular epithelial cells, edema of the lamina propria, and translucent vacuoles at the bases of the remaining epithelial cells (Fig. 2C), as did preparations kept in TP culture medium that were exposed to the same dose of amebal lysates (Navarro-García et al. 1993). Treatment with 100000 lysed trophozoites provoked the most severe damage, characterized by destruction of the mucosal cells along interglandular zones and abundant vacuoles at the bases of the remaining epithelial cells (Fig. 2D).

Electron microscopy. The magnitude of the electrophysiologic effects also correlated with the extent of the ultrastructural lesions detected. Untreated samples appeared normal under the electron microscope, their epithelia consisting of columnar cells with oval nuclei (Fig. 3A). In contrast, the smallest dose caused lesions consisting mostly of vacuolation at the bases of the epithelial cells (Fig. 3B). The epithelia of preparations treated with the intermediate dose had lost their intercellular junctions and the cells were shortened, showing nuclei deformed from an oval to a circular shape (Fig. 3C). Contrasting with their normal appearance in untreated preparations, microvilli were fragmented or disappeared from treated preparations. The most severe damage, caused by the highest dose, consisted of massive desquamation and destruction of interglandular epithelial cells as well as loss of intercellular junctions and microvilli fragmentation in the remaining epithelial cells (Fig. 3D).

Temporal course of the morphologic damage

In view of the correlation of the morphologic and electrophysiologic lesions with the lysate doses used, we decided to analyze the temporal course of the damaging process. For this purpose we used three simultaneously mounted colon samples derived from the same rabbit

Fig. 2A–D. Effect of variable doses of *E. histolytica* HM1 trophozoite lysates on the morphology of colon preparations. The colon segments were fixed with glutaraldehyde and embedded in Epon, and semithin sections were stained with toluidine blue. **A** Untreated preparation kept for 120 min. **B–D** Preparations treated for 80 min with 25000 (**B**) or 50000 lysed trophozoites (**C**) and for 60 min with 100000 lysed trophozoites (**D**)

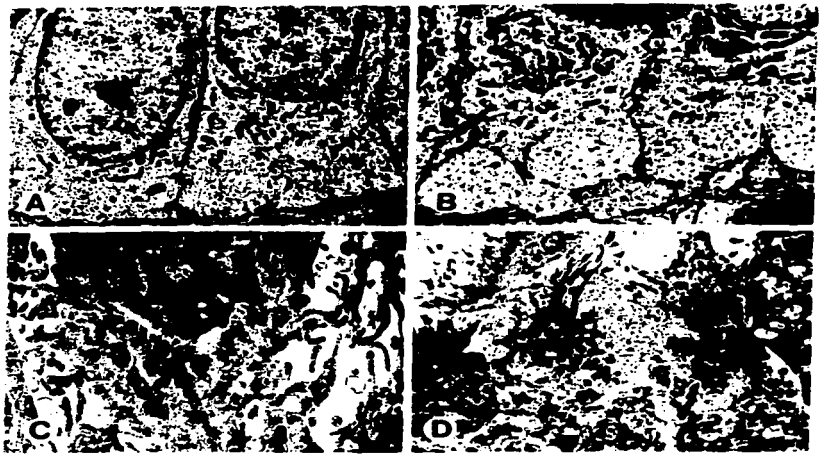

Fig. 3A–D. Ultrastructural damage to the colon mucosa in preparations treated with variable doses of *E. histolytica* HM1 trophozoite lysates. **A** Normal epithelial cells of an untreated preparation kept for 120 min. **B–D** Damaged epithelial cells of preparations treated for 80 min with 25000 (**B**) or 50000 lysed trophozoites (**C**), and for 60 min with 100000 lysed trophozoites (**D**)

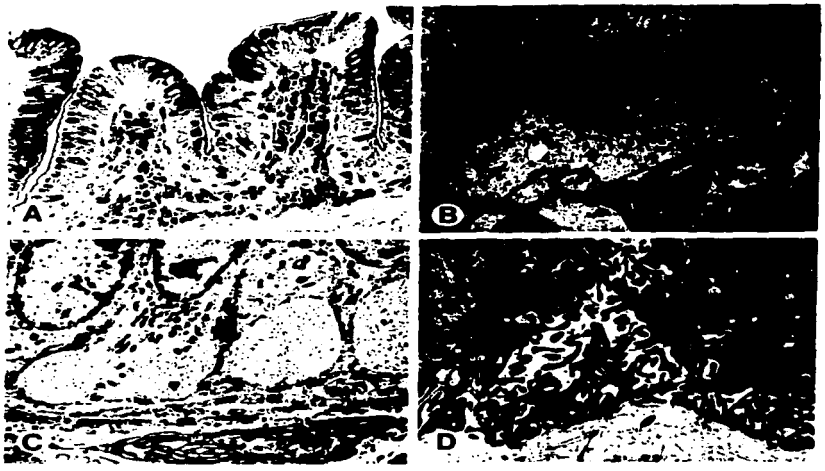

Fig. 4A–D. Sequential deterioration of colon preparations treated with a fixed dose of *E. histolytica* HM1 trophozoite lysates. Three colon segments from the same animal were mounted in Ussing chambers. At 15 min after the addition of 50000 lysed trophozoites, the first preparation was removed and fixed with glutaraldehyde; the second and third preparations were treated for 40 and 80 min, respectively. **A** Light microscopy and **B** electron microscopy of a preparation treated for 15 min. **C** Electron microscopy of a preparation treated for 45 min. **D** Electron microscopy of a preparation treated for 80 min

and treated them with an identical dose of 50000 lysed trophozoites. After 15 min of exposure, the first preparation was removed from the Ussing chamber and fixed with glutaraldehyde; the second and third preparations were exposed for 40 and 80 min before being removed and fixed. The mean PD_0 and Isc_0 values were 7 mV and 40 $\mu A/cm^2$, respectively.

Semithin sections of the preparations treated for 15 min showed the least damage, consisting of small subnuclear vacuoles at the bases of epithelial cells, which

under the optical microscope appeared to separate the epithelial layer from the lamina propria (Fig. 4A). Exposure for 40 min caused edema of the lamina propria, whereas after 80 min, when PD and Isc had just reached nil values, there were increases in the desquamation of the interglandular epithelium, the edema of the lamina propria, and the formation of vacuoles at the bases of the remaining epithelial cells (see Fig. 2C).

Ultrastructural damage was detected mainly in the epithelial cells. A 15 min exposure induced edema that progressed to incipient subnuclear vacuolation in the basal portion of epithelial cells (Fig. 4B). At 40 min of exposure, the subnuclear vacuoles were larger (Fig. 4C); at 80 min the mucosal layer was detached from the lamina propria and intercellular junctions were lost among the remaining epithelial cells (Fig. 4D).

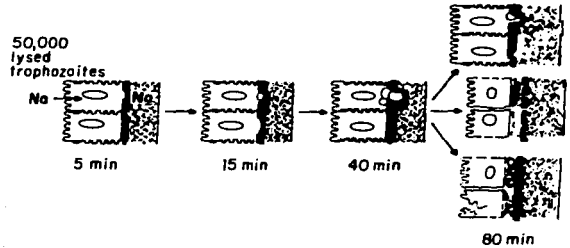

Fig. 5. Schematic drawing of the temporal course of the lesions caused by 50000 *E. histolytica* HM1 lysed trophozoites to the mucosal cells of rabbit colon preparations

Discussion

In previous studies we have shown that 50000 lysed *Entamoeba histolytica* HM1 trophozoites added to the luminal side of rabbit colon preparations kept either in basal culture medium for amebae (López-Revilla et al. 1992) or in Ringer's solution (Navarro-García et al. 1992) induce an immediate fall of PD, Isc, and R values, whereas the same dose of *E. invadens* PZ (a strain virulent in reptiles) trophozoite lysates has undetectable electrophysiologic effects. We have also reported that the earliest microscopic signs of damage to intestinal segments exposed to *E. histolytica* lysates is the presence of translucent vacuoles at the bases of epithelial cells, later followed by the detachment of the epithelial layer from the lamina propria and the destruction of interglandular epithelial cells (Navarro-García et al. 1993).

In this work we analyzed the influence that variable doses of and times of exposure to *E. histolytica* lysates have on rabbit colon. We found that the magnitude of the electrophysiologic effects, mainly on PD and R values, depended on the dose and correlated with the severity of the microscopic lesions observed. The smallest dose (25000 lysed trophozoites) induced the formation of small translucent vacuoles that under the light microscope appeared to be located between the epithelial layer and the lamina propria; with the electron microscope we located such vacuoles under the nuclei at the bases of epithelial cells. At the intermediate dose (50000 lysed trophozoites), the epithelial layer started to separate from the lamina propria and incipient necrosis, mostly of interglandular epithelial cells, also occurred; under the electron microscope these preparations had shortened epithelial cells that were devoid of intercellular junctions and had deformed nuclei and fragmented microvilli. The highest dose (100000 lysed trophozoites) caused massive desquamation and destruction of interglandular epithelial cells. The start of microvillar fragmentation coincided with the beginning of the decay of PD and Isc, properties that are known to depend on the apical transport of Na^+ by microvillar pumps and channels (Frizzell et al. 1976). The loss of intercellular junctions coincided with the beginning of R decay, probably due to the free passage of ions through the paracellular route.

Having established the correlation between the dose of lysates and the electrophysiologic and morphologic changes, we determined the temporal course of production of the morphologic lesions in preparations treated with a unique (intermediate) dose of lysates. After 15 min there was incipient vacuole formation at the basal portion of epithelial cells. After 40 min the vacuoles had increased in size and intercellular junctions were lost. After 80 min, when PD and Isc had just reached nil values, there were large subnuclear vacuoles at the bases of the interglandular epithelial cells, the loss of tight junctions was complete, and there was also a massive loss of interglandular epithelial cells.

In Fig. 5 we attempt to summarize the sequence of the physiologic and morphologic changes occurring in the colon mucosa in preparations treated with the intermediate dose of *E. histolytica* lysates. The lysates probably first blocked mucosal to serosal Na^+ transport as reflected by early PD and Isc decay, perhaps associated with damage to the microvilli, and then caused the formation of cytoplasmic vacuoles in the basal portion of the epithelial cells; these vacuoles may be associated with the water transport blockage known to accompany Na^+ transport inhibition (Sandle et al. 1990). The loss of intercellular epithelial junctions after 60 min of treatment coincided with the annulment of transmural resistance.

The structural lesions described herein are similar to those previously seen in human biopsies and in experimental intestinal amebiasis. For instance, in human biopsies Prathap and Gilman (1970) found mucosal edema, epithelial cell shortening, and interglandular lesions, whereas in guinea pigs Takeuchi and Phillips (1975) observed a shortening and disappearance of microvilli, a loss of tight junctions, and interglandular epithelial cell damage. On the other hand, Knight and co-workers (1974) showed that during the early interaction of amebae with RK13 monolayer cells, electron microscopic damage was not detectable in the plasma membrane but occurred in the cytoplasm, consisting of vacuolation and

a loss of mitochondrial crests. Orozco et al. (1982) have found that on Madin Darby canine kidney (MDCK) epithelial cell monolayers, amebae cause a loss of transepithelial resistance that correlates with the loss of tight junctions evidenced by electron microscopy with macromolecular markers.

The experimental approach described herein allowed us to detect the earliest electrophysiologic and morphologic lesions described to date in experimental intestinal amebiasis, i.e., PD and Isc decay and vacuolation at the bases of epithelial cells. Our model also has several advantages over other in vitro and animals models: (a) it is closer to intestinal amebiasis because the target is a full-thickness intestinal segment and not cultured cells, (b) the electrophysiologic properties of the colon segments exposed to trophozoite lysates may be correlated with their morphologic and biochemical changes, (c) immediate location of the microscopic lesions is possible because the intestine-lysate interaction takes place in a specified minimal area, and (d) the results are reproducible, in contrast to those obtained in animals inoculated intracecally, which are inconsistent and take several days to develop. These features suggest that our model will be useful in expanding our understanding of the pathogenesis of intestinal amebiasis.

Acknowledgements. We wish to acknowledge the critical review of the manuscript by Dr. Adolfo Martinez-Palomo and the technical help of Alicia Ramirez and Jaime Escobar. This work was partially supported by CONACYT (Mexico), the MacArthur Foundation (USA), and a CONACYT scholarship (granted to F.N.G.).

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CAPITULO IV

Entamoeba histolytica: Increase of Enterotoxicity and of 53- and 75-kDa Cysteine Proteinases in a Clone of Higher Virulence

PAGINACION VARIA

COMPLETA LA INFORMACION

EXPERIMENTAL PARASITOLOGY 80, 361–372 (1995)

Entamoeba histolytica: Increase of Enterotoxicity and of 53- and 75-kDa Cysteine Proteinases in a Clone of Higher Virulence

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*Departments of *Cell Biology, ‡Experimental Pathology, and §Pharmacology and Toxicology, CINVESTAV-IPN, Ap. postal 14-740, 07000 Mexico DF, Mexico; and †Department of Public Health, Facultad de Medicina-UNAM, Ciudad Universitaria, 04510 Mexico DF, Mexico*

NAVARRO-GARCÍA, F., CHÁVEZ-DUEÑAS, L., TSUTSUMI, V., POSADAS DEL RÍO, F., AND LÓPEZ-REVILLA, R. 1995. *Entamoeba histolytica:* Increase of enterotoxicity and of 53- and 75-kDa cysteine proteinases in a clone of higher virulence. *Experimental Parasitology*, 80, 361–372. We compared the enterotoxicity and cysteine proteinases (CP) of the low-virulence *Entamoeba histolytica* HM1 strain with the highly virulent 1659 clone, derived from HM1 by hamster liver passages. Enterotoxicity of 50,000 freeze–thawed trophozoites was determined on 0.28-cm^2 intestinal segments mounted in Ussing chambers; CP activity of Nonidet-P40 amebal lysates was assayed by gelatin-sodium dodecyl sulfate–polyacrylamide gel electrophoresis and carbobenzoxy-L-arginine-L-arginyl-*p*-nitroaniline, a CP-specific substrate. Treatment of gerbil cecum segments with amebal lysates caused an immediate fall of their electrophysiologic properties (potential difference, short-circuit current, and transmural resistance) whose decay rates were clearly faster with 1659 than with HM1 lysates. Nonimmune and immune antiamebic human sera and the CP-specific inhibitor E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane) prevented the fall of the electophysiologic properties. Gelatinases, less active in HM1 than in 1659 trophozoites, were better preserved in lysates containing 10 m*M* *p*-hydroxymercuribenzoate (pHMB) to prevent autoproteolysis: in lysates without pHMB nearly no gelatinase bands were observed in HM1 samples, whereas intense 30K, 35K, 44K, and 75K bands were seen in 1659 samples; in lysates with pHMB only 53K and 75K bands were found that were much more intense in 1659 samples, 75K being barely visible in HM1 samples. The overall CP activity was 17 times higher in 1659 than in HM1 lysates, was inhibited by E-64 (mean inhibitory dose, 20 μM), was stimulated by 2-mercaptoethanol (ME) 3.7 times in HM1 and 2.4 times in 1659 lysates, and was reactivated by ME in lysates containing pHMB. Most of the CP activity in HM1 lysates sedimented at 15,600*g* but predominated in 1659 supernatants. The increase of *E. histolytica* virulence thus correlates with a remarkable increase both of *in vitro* enterotoxicity and of two CPs (53K and 75K), suggesting that these proteinases are significant pathogenicity factors. © 1995 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Entamoeba histolytica;* Virulence; Intestinal amebiasis; Ussing chamber; Short-circuit current (Isc); Potential difference (PD); Transmural resistance; Gerbil cecum; Proteases; Cysteine-proteinases (CP).

INTRODUCTION

Entamoeba histolytica trophozoites may colonize and destroy the mucosa of the large intestine and damage other organs, especially the liver (Pérez-Tamayo 1986). Amebal pathogenicity, multifactorially determined, is due to the invasiveness of trophozoites (Espinosa-Cantellano and Martínez-Palomo 1991). Although the factors that determine the invasiveness of the parasite have not been unequivocally identified, damage and degeneration of intestinal epithelial cells and dissolution of the mucosal basement membrane clearly indicate that histolytic factors play a major role in amebal virulence (Takeuchi and Phillips 1975, Ghadirian and Meerovitch 1978, Navarro-García *et al.* 1993a).

Cysteine-proteinases (CP) with gelati-

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nase activity predominate over other proteases in *E. histolytica* trophozoites (Scholze and Schulte 1988; Keene *et al.* 1986) and appear to be significant pathogenic factors (Gadasi and Kobiler 1983; Lushbaugh *et al.* 1985; Gadasi and Kessler 1983; Reed *et al.* 1989; McKerrow *et al.* 1993). However, Montfort *et al.* (1993) recently have put in doubt the contribution of proteases to amebal virulence.

In this work we compared the enterotoxicity and the CPs of a low-virulence *E. histolytica* strain (HM1) with those of a highly virulent clone (1659) derived from the first and selected by its ability to produce large liver abscesses in hamsters. Clone 1659 trophozoites were more toxic and damaging to the mucosa of intestinal preparations mounted in Ussing chambers and had a remarkably increase of CP activity due to gelatinases of 53 kilodaltons (kDa) and 75 kDa.

Materials and Methods

Parasites

E. histolytica HM1 trophozoites, whose origin has been described (López-Revilla and Gómez-Domínguez 1985), have been kept more than 15 years in axenic culture in our laboratory, first in TPS-1 medium (Diamond 1968) and then in TYI-S-33 medium (Diamond *et al.* 1978). Under Results we describe how the highly virulent clone 1659 was derived from the HM1 strain.

Trophozoite Lysates and Fractions

The lysates used in electrophysiologic experiments were obtained from log phase trophozoites, washed by centrifugation in cold basal TYI medium, and stored in liquid nitrogen as pellets with 5×10^5 trophozoites. At the beginning of each experiment, the pellets were diluted with 0.5 ml of Ringer's solution (López-Revilla *et al.* 1992a), and amebal lysates obtained through a freeze–thawing cycle were mixed with 2 ml of the same diluent at 37°C to obtain suspensions containing 20,000 trophozoites/ml (Navarro-García *et al.* 1993b).

Gelatinases and CP activity were assayed in lysates obtained from log phase trophozoites that were washed twice by centrifugation at 600*g* with cold basal TYI medium and then dissolved with lysis buffer, consisting of 1% Nonidet-P40 (NP40) in 12 m*M* sodium phosphate, pH 6.8 (López-Revilla *et al.* 1992a); when indicated, the lysis buffer was supplemented with three protease inhibitors to the following final concentrations: 2 m*M* sodium ethylenediaminotetraacetate, 2 m*M* phenylmethylsulfonyl fluoride, and 10 m*M* *p*-hydroxymercuribenzoate (pHMB), to respectively block metallo-, serine-, and cysteine-proteinases. From lysates obtained with NP-40 called total extracts, supernantant and pellet fractions were obtained by centrifugation for 5 min at 15,600*g*.

Electrophysiologic Experiments

The large intestine was removed from adult males under sodium pentobarbital anesthesia. Segments of the ascending colon from New Zealand rabbits or the cecum from gerbils (*Meriones unguiculatus*) were placed in ice-cold Ringer's solution for mammals at 4°C and gassed with an O_2–CO_2 (95%:5%) mixture. Each segment was cut open along its mesenteric border, rinsed clean of luminal contents, and extended and kept in gassed Ringer's solution. Full-thickness segments were divided in two and mounted between the circular openings (diameter = 6 mm) of two adjacent Ussing hemichambers. Each hemichamber was filled with 2.5 ml of gassed Ringer's solution and kept at 37°C while bubbling with the O_2–CO_2 mixture in both hemichambers. Transmural potential difference (PD) and short-circuit current (Isc) values were recorded at 5-min intervals by means of a voltage clamp; transmural resistance (R) values were obtained from PD/Isc ratios at each time point; only reliable R values derived from Isc values higher than 2 $\mu A/cm^2$ were plotted (López-Revilla *et al.* 1992b; Navarro-García *et al.* 1993a,b).

Suspensions containing 50,000 freeze–thawed trophozoites in 2.5 ml were added to the mucosal hemichamber of preparations that had been mounted for 5 min; fresh Ringer's solution at 37°C was then added to the serosal hemichamber and both hemichambers were gassed with O_2–CO_2. Initial decay rates were calculated by regression of the PD and Isc curves during the first 20 min of exposure. Statistical analysis was performed using the Student's t test on data subjected to arcsin transformation (Navarro-García *et al.* 1993a,b).

At the end of electrophysiologic experiments, the preparations were removed from the Ussing chambers and immediately fixed for 1 hr with 2.5% glutaraldehyde dissolved in 0.1 *M* sodium cacodylate, pH 7.4. Segments were cut into 1-mm-thick sections, fixed for a further 2 hr, postfixed with osmium tetroxide, dehydrated with ethanol at increasing concentrations, and finally embedded in Epon 812. Semithin (1-μm-thick) sections were stained with toluidine blue to be examined with a light microscope (Navarro-García *et al.* 1993b).

The nonimmune and immune (from amebal liver abscess convalescents) human sera used in electrophys-

iologic experiments had been titered by indirect hemagglutination with *E. histolytica* antigen. The CP-specific inhibitor E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane) was obtained from Sigma Chemical Co. (St. Louis, MO). Before being added to the mucosal hemichamber of gerbil cecum preparations, the lysates used (obtained by freeze–thawing 0.5-ml suspensions containing 50,000 trophozoites) were mixed and incubated for 1 min with 250 μl of either nonimmune, immune serum (1:10 dilution), or 40 μl of 32 mM E-64; fresh Ringer's solution at 37°C was added to the serosal hemichamber. Control preparations were treated with lysates that had not been incubated with serum, as described elsewhere (Navarro-García *et al.* 1993b).

Proteinase Assays

Gelatinases. HM1 and 1659 lysates were subjected to electrophoresis in substrate gels to assay their gelatinases *in situ* (López-Revilla *et al.* 1992a). The lysates were obtained with NP-40 alone or in the presence of the protease inhibitors; after electrophoresis the gels were washed three times with 100 m*M* Tris–HCl, 150 m*M* NaCl, pH 7.4, at 4°C and incubated at 37°C in 75 m*M* Tris–HCl, 150 m*M* NaCl, pH 7.4, alone or with 110 m*M* 2-mercaptoetanol (ME). After staining with Coomassie brilliant blue, clear bands or zones could be seen in the gels where active gelatinases were located.

Cysteine-proteinases. The molar extinction coefficient of *p*-nitroaniline (pNA)—hydrolysis product of ZRRpNA (carbobenzoxy-L-arginine-L-arginyl-*p*-nitroanilide), a dipeptidyl substrate specific for CP used to assay *Entamoeba* lysates (R. López-Revilla, L. Chávez-Dueñas, F. Posadas del Río, submitted for publication)—at 405 nm was 4860 in EIA Costar microplates containing 200 μl volumes per well. Reaction mixtures containing 0.2 m*M* ZRRpNA and either total extract, supernatant, or pellet fractions from 10^6 NP-40-lysed trophozoites were assayed at room temperature, in the absence or in the presence of 110 m*M* ME, and hydrolysis rates were determined by measuring the increase in absorbance at 405 nm (A_{450}) with a BioRad 450 ELISA reader. CP-specific activity is expressed as pmoles of pNA produced per min per μg of amebal protein (R. López-Revilla *et al.*, submitted for publication).

RESULTS

Selection of the More Virulent Clone, 1659

One week after intrahepatic inoculation of hamsters, HM1 trophozoites produced small whitish spots on the liver surface or isolated lesions less than 2 mm in diameter (Fig. 1A) which disappeared spontaneously 1 week later.

Trophozoites of increased virulence were selected by culturing samples of the largest abscesses produced after each of four successive liver passages of HM1 trophozoites. From them, clone 1659 trophozoites were selected by diluting and culturing twice before propagating and cryopreserving them in liquid nitrogen. One week after their inoculation, clone 1659 trophozoites produced multiple abscesses involving approximately 40% of the liver (Fig. 1B).

Effects of Amebal Lysates on Gerbil Cecum

Electrophysiologic effects. The PD and Isc values of full-tickness gerbil cecum preparations fell spontaneously to nil values in about 160 min, and their initial decay rates were -0.0080 mV min^{-1} for PD and -0.0096 μA cm^{-2} min^{-1} for Isc. Treatment of gerbil cecum preparations with HM1 lysates caused immediate and significantly different decay rates for PD and Isc ($P < 0.05$), which reached nil values 110 min after lysate addition (Figs. 2A and 2B); the initial decay rates were -0.0117 mV min^{-1} for PD and -0.0122 μA cm^{-2} min^{-1} for Isc and thus 1.5 and 1.3 times faster than the corresponding spontaneous decay rates.

In cecum preparations treated with 1659 lysates the values of both electrical properties were nil 75 min after lysate addition (Figs. 2A and 2B); the initial decay rates were -0.0126 mV min^{-1} for PD and -0.0134 μA cm^{-2} min^{-1} for Isc and thus 1.5 and 1.4 times faster than those of HM1-treated preparations.

Although both HM1 and 1659 lysates caused electrical resistance to fall, the initial decay rates were 1.3 times faster in preparations treated with 1659 lysates (-0.0061 Ω cm^2 min^{-1}) than with HM1 lysates (-0.0047 Ω cm^2 min^{-1}) and thus 3.8 and 2.9 times faster respectively, than the spontaneous decay rate of -0.0016 Ω cm^2 min^{-1} (Fig. 2C).

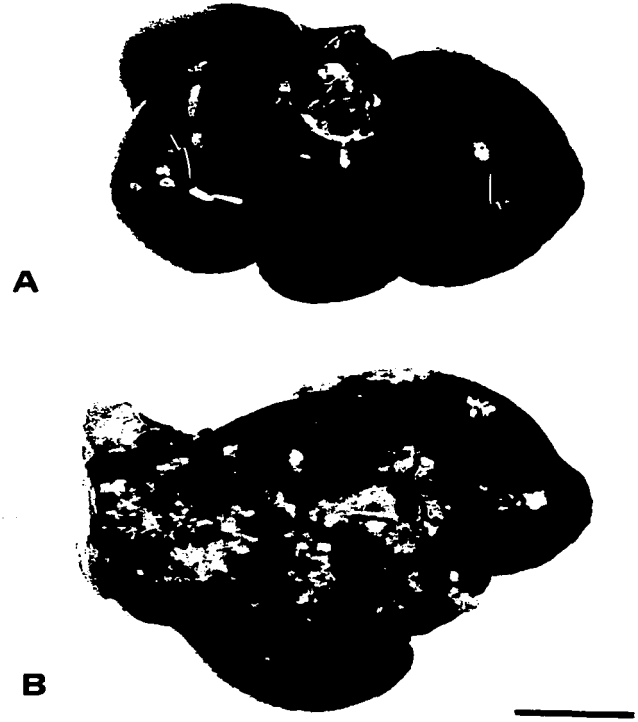

FIG. 1. Livers from hamsters inoculated with 10^6 *E. histolytica* trophozoites. (A) Strain HM1. Small isolated lesions (arrows) are seen on the liver surface. (B) Clone 1659. Extensive, irregular necrotic lesions affect almost half of the liver. Bar = 10 mm.

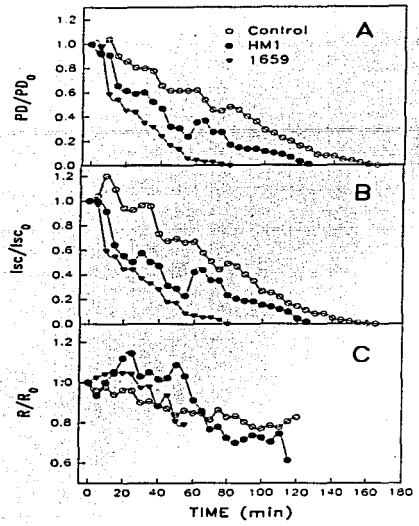

FIG. 2. Effects of *E. histolytica* HM1 and 1659 trophozoite lysates on the electrophysiologic properties of gerbil cecum preparations. Both hemichambers contained Ringer's solution gassed with 95% O_2–5% CO_2; 5 min after each preparation had been mounted, 50,000 freeze–thawed HM1 or 1659 trophozoites were added to the luminal hemichamber. Initial values (mean ± SE) of potential difference, short-circuit current, and transmural resistance were PD_o = 2.9 ± 0.5 mV, Isc_o = 40.5 ± 10.4 μA/cm², and R_o = 73.5 ± 4.8 Ω cm², respectively. The symbols represent average normalized values of PD/PD_o, Isc/Isc_o and R/R_o ratios, i.e., (each time value)/(initial value); n = 4 for HM1, n = 5 for 1659.

Morphologic effects. Even after having been mounted for 180 min in Ussing chambers, untreated gerbil cecum preparations appeared almost normal under the light microscope (Fig. 3A), contrasting with HM1- and 1659-treated preparations from the same animal, whose mucosae were clearly damaged. The lesions caused by 115 min exposure to HM1 lysates consisted of small translucent vacuoles located between the epithelial layer and the lamina propria and edema of the lamina propria (Fig. 3B). In contrast, preparations exposed only 75 min to 1659 lysates had much more intense lesions consisting of small translucent vacuoles under the epithelial layer, loss of continuity of the interglandular epithelium, damage to the epithelial cells, and edema of lamina propria (Fig. 3C). The magnitude of the microscopic lesions also correlated with the electrophysiologic damage caused by HM1 and 1659 lysates.

Proteinases

Gelatinases. Only two tenuous gelatinase bands of 44 and 30 kDa were detected in HM1 lysates obtained without inhibitors and subjected to electrophoresis in substrate gels incubated without ME (Fig. 4, lane A). In contrast, 1659 lysates without inhibitors had two wide zones of activity around 75 kDa (75K) and 44 kDa (44K) and two intense bands of 35 kDa (35K) and 30 kDa (30K) near the electrophoretic front (Fig. 4, lane B). In gels incubated with ME, HM1 lysates showed two 30K and 44K tenuous bands (Fig. 4, lane E), whereas 1659 lysates produced the same but wider zones of activity around 75K, 44K, 35K, and 30K (Fig. 4, lane F).

Substrate gels run with HM1 lysates with inhibitors and incubated without ME had a predominant 53K band and two 75K and 30K tenuous bands (Fig. 4, lane C), whereas gels with 1659 lysates showed two 75K and 53K intense bands and a much less visible 30K one (Fig. 4, lane D). When gels with these HM1 lysates were incubated with ME the 53K band also predominated although it was much wider than in gels incubated without ME, and three less intense 75K, 40K, and 35K bands also appeared (Fig. 4, lane G); gels run with 1659 lysates had seven bands: four diffuse major ones whose decreasing order of intensity was 53K, 75K, 40K, and 35K and three tenuous

FIG. 3. Effect of *E. histolytica* HM1 and 1659 trophozoite lysates on the mucosa of gerbil cecum. Cecum segments employed in electrophysiologic experiments were fixed with glutaraldehyde and embedded in Epon; semithin sections were obtained and stained with toluidine blue. (A) Untreated cecum preparation kept for 180 min in the Ussing chamber. (B) Preparation exposed 115 min to HM1 lysates. (C) Preparation exposed 75 min to 1659 lysates.

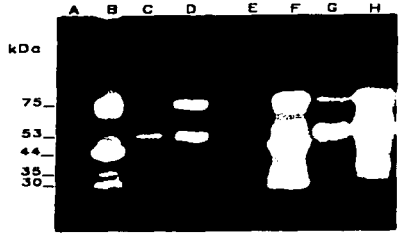

FIG. 4. Differences in the molecular weight and activities of the gelatinases of *E. histolytica* HM1 and 1659 lysates. The lysates were obtained with 100 μl of 0.1% NP-40 in 12 m*M* sodium phosphate buffer (with or without proteinase inhibitors) per 10^6 trophozoites; they were then subjected to electrophoresis in substrate gels (0.1% gelatin-polyacrylamide–SDS) by applying 20 μl samples per lane. After the electrophoretic run, the gels were washed, incubated 45 min at 37°C without or with 110 m*M* 2-mercaptoethanol, and stained with Coomassie blue. (Lane A), HM1, and (lane B), 1659 lysates without inhibitors and ME; (lane C), HM1, and (lane D), 1659 lysates with inhibitors and without ME; (lane E), HM1, and (lane F), 1659 lysates without inhibitors and with ME; (lane G), HM1, and (lane H), 1659 lysates with inhibitors and with ME.

ones around 112, 136, and 159 kDa (Fig. 4, lane H).

Cysteine-proteinases. Total CP activity of 1659 lysates was much larger than in HM1 lysates: 17 times higher in assays without ME, and 25 times higher in assays with ME (Table I). The distribution of CP activity in the fractions obtained by centrifugation of 1659 and HM1 lysates was different; it predominated in the 1659 supernatants (81% of total activity in assays without ME, 66% in assays with ME) and the HM1 pellets (69% of total activity in assays without ME, 76% with ME; Table I).

The magnitude of CP stimulation by ME depended on the source of the lysates and on the fractions used. Total activity was stimulated 3.7 and 2.4 times in 1659 and HM1 lysates; 1659 and HM1 supernatants were stimulated 3 and 4.5 times, whereas the pellets were stimulated 8.6 and 2.7 times.

TABLE I
CP Activity in Total Extracts (TE), Supernatants (S), and Pellets (P) of HM1 and 1659 Trophozoites

Activity	Fraction	HM1		1659	
		−ME[a]	+ME[b]	−ME	+ME
Total[c]	TE	36.2	88.0	603.6	2232.7
	S	10.1	45.2	488.5	1480.8
	P	25.1	67.0	78.6	677.2
Specific[d]	TE	6.3	16.8	137.4	511.2
	S	1.7	11.1	127.2	387.8
	P	5.3	16.1	33.2	294.5

[a] Incubation without ME.
[b] Incubation with 110 m*M* ME.
[c] pmoles pNA min^{-1} (10^4 amebae)$^{-1}$.
[d] pmoles pNA min^{-1} (μg protein)$^{-1}$.

The CP-specific activity also increased greatly in clone 1659 trophozoites. The specific activities of 1659 lysates and their supernatant and pellet fractions assayed without ME were, respectively 17, 48, and 3 times higher than those of HM1 lysates and their fractions. In assays with ME the specific activity values of 1659 lysates and their fractions were 30, 34, and 18 times higher than those of HM1 lysates and their fractions.

Both nonimmune human serum and E-64 inhibited the overall CP activity of amebal lysates in a concentration-dependent manner. The mean inhibitory dose for E-64 was 20 μ*M*, whereas the inhibition (91%) caused by 10% serum was equivalent to that produced by 240 μ*M* E-64.

Prevention by Human Serum of the Fall of Electrophysiologic Properties Caused by Amebal Lysates

We have already shown that HM1 trophozoite lysates cause an immediate fall of the electrophysiologic properties of rabbit colon preparations, whose rate of decay is dose- and time-dependent and correlates with the microscopic damage caused to the mucosa, mainly to epithelial cells (Navarro-García *et al.* 1993a,b).

In this work we asked if human serum containing antiamebic antibodies could protect against the effects of HM1 lysates on the electrical properties of gerbil cecum. We found that electrical resistance decreased to less than half its initial value in preparations treated with amebal lysates for 60 min (Fig. 5C), that preincubation of amebal lysates with nonimmune serum or E-64 prevented the fall of electrical resistance (Fig. 5C), PD, and Isc (Fig. 5A and 5B), and that immune serum had the same effect (data not shown).

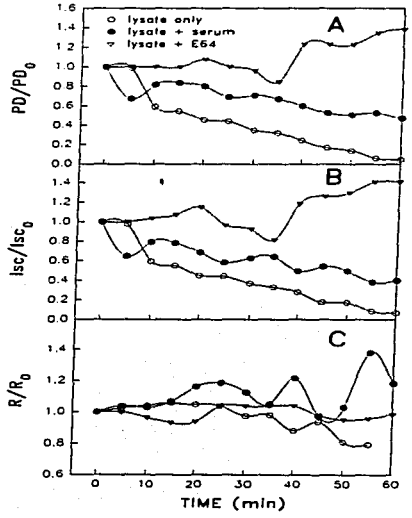

FIG. 5. Prevention by nonimmune human serum and E-64 of the fall of electrophysiologic properties caused by 1659 lysates on gerbil cecum preparations. Two preparations from the same gerbil were mounted in Ussing chambers. During the first 5 min of the experiment both chambers contained gassed Ringer's solution; 50,000 freeze–thawed trophozoites were added to the luminal hemichamber of the first chamber, whereas the same number of freeze–thawed trophozoites that had been preincubated with either 10% nonimmune human serum or 32 μM E-64 was added to the luminal hemichamber of the second chamber.

DISCUSSION

E. histolytica virulence may decrease in cultured trophozoites (see for instance Montfort *et al.* 1993; Tsutsumi *et al.* 1992) and be recovered in parasites selected from the abscesses that they cause after serial passages in hamster liver (Diamond *et al.* 1974, Tsutsumi *et al.* 1992). Under *in vitro* culture conditions, the expression of amebal genes encoding pathogenic factors might decrease if it does not represent a selective advantage; the increase of virulence through liver passage might depend on regaining the gene expression lost in the parental attenuated strains. To study pathogenic mechanisms it would be safer to compare more virulent trophozoite variants with their attenuated progenitors than to follow the usual and opposite approach of comparing attenuated amebal variants, defficient in functions selected *a priori*, with the more virulent progenitor strains (Orozco *et al.* 1983); the changes in such attenuated variants may be due to metabolic or structural deficiencies unrelated to the pathogenic mechanisms.

In this work we have found that the increased virulence of *E. histolytica* clone 1659 trophozoites, selected after four hamster liver passages (Fig. 1), is accompanied by a remarkable increase of their enterotoxicity and of the activity of two CPs, 53K and 75K, the second of which appears not to have been identified before.

Intestinal amebiasis is important because it is much more frequent than the extraintestinal form of amebiasis, is the source of infection and the origin of extraintestinal cases, and would be the natural target in preventing the infection by vaccination. To study the pathogenic mechanisms and local immunity in intestinal amebiasis we have developed an *in vitro* experimental model in which *E. histolytica* trophozoite lysates

produce an immediate fall of the electrophysiological properties in Ussing-chambered preparations of the large intestine of rabbits (Navarro-García *et al.* 1993b) and rats (Navarro-García *et al.* 1994). In the present work we used gerbil cecum preparations because only in these animal species both intestinal and hepatic amebiasis can be experimentally produced (Chadee and Meerovitch 1985, Shibayama-Salas *et al.* 1992a,b). The magnitude of the morphologic and electrophysiologic lesions caused on gerbil cecum by amebal lysates correlated with parasite virulence, because 1659 lysates provoked an immediate deterioration of the electrical properties of intestinal preparations with initial decay rates 1.5 times faster than those caused by HM1 lysates (Fig. 2). Furthermore, the microscopic damage to the cecal mucosa was more severe with 1659 than with HM1 lysates and the magnitude of the microscopic lesions correlated with the electrophysiologic effects (Fig. 3), as shown for rabbit colon preparations (Navarro-García *et al.* 1993a,b).

CPs are the most active of *E. histolytica* proteinases (Scholze and Schulte 1988; Keene *et al.* 1986; Pérez-Montfort *et al.* 1987) and appear to be essential for the parasites because their specific inhibition greatly affects trophozoite growth (De Meester *et al.* 1990), and their molecular weights have been found to range between 16 and 56 kDa (Lushbaugh *et al.* 1985; Scholze and Schulte 1988; Luaces and Barret 1988; Keene *et al.* 1986). They are the major cause of autoproteolysis in amebal lysates obtained with SDS (Ávila *et al.* 1985; López-Revilla *et al.* 1992b), are stimulated by ME (López-Revilla *et al.* 1992b) and their gelatinase activity allows their *in situ* identification in substrate gels (Keene *et al.* 1986; Reed *et al.* 1989; López-Revilla *et al.* 1992b). The 56K proteinase appears to be secreted, is antigenic in patients with invasive amebiasis (Reed *et al.* 1989) and appears to be a significant pathogenic factor (McKerrow *et al.* 1993). CPs exert a "cytotoxic" activity consisting in the detachment of surface-cultured animals cells (Gadasi and Kessler 1983; Lushbaugh *et al.* 1985) that correlates with the virulence of *E. histolytica* isolates, degrade extracellular matrix proteins (Keene *et al.* 1986; Luaces and Barrett 1988; Schulte and Scholze 1989) and other proteins *in vitro* (Lushbaugh *et al.* 1985; Scholze and Schulte 1988), and may help in the spreading of the parasites by attacking the proteins that anchor mucosal epithelial cells to the basement membranes and those forming intercellular junctions (Reed *et al.* 1989).

After we found that gelatinases were much more active in 1659 than in HM1 trophozoites, we confirmed our previous observations that the presence of proteinase inhibitors (especially pHMB) prevents the autoproteolysis induced by SDS and consequently preserves the larger proteinases and decreases the number of active gelatinases (López-Revilla *et al.* 1992b). We also confirmed the stimulatory effect of ME on both CP (Keene *et al.* 1986; Lushbaugh *et al.* 1985; López-Revilla *et al.* 1994) and gelatinase activity (López-Revilla *et al.* 1992b).

Although *E. histolytica* genes encoding CPs of 27 and 30 kDa have already been cloned and sequenced (Eakin *et al.* 1990; Tannich *et al.* 1991), the predominance of 75K and 53K in 1659 lysates where proteolysis was prevented suggests that these proteinases are different and may be encoded by other genes. The greater relative increase of 75K in 1659 trophozoites and its detection in HM1 lysates only when proteolysis was prevented suggest that this CP is different to 53K, which predominates in HM1 lysates. Even though the sizes of the two minor proteinases found in 1659 lysates (30K and 35K) are close to those of the already cloned CP genes, they appear to derive mainly from larger precursors because (1) they were detected in HM1 lysates obtained with inhibitors but not in their ab-

sence, (2) ME stimulation made 35K the predominant species at the same time that 53K was more tenuous, and (3) 30K increased in HM1 and 1659 lysates when autoproteolysis was not prevented.

The higher virulence of *E. histolytica* 1659 trophozoites correlated with a notable increase of CPs, including a change in their subcellular distribution. In HM1 lysates most of the CP activity appeared to be due to 53K (Fig. 4, lane C) located in the pellet fraction (Table I), and therefore associated to NP-40-insoluble components. The CP activity increase of 17–25 times in 1659 lysates was mainly due to 53K and 75K gelatinases, although 75K had the greatest relative increment (Fig. 4, lane D). In 1659 lysates 75K was more intense than 53K, was located mainly in the supernantant fraction (Table I), and was barely detected in HM1 lysates (Fig. 4, lane C) where the 53K band was more intense than the 75K. These results suggest that the increased virulence of 1659 trophozoites may be due to the increase of 75K and 53K CPs, which might be encoded by genes different from those already cloned that encode 27K and 30K CPs (Eakin *et al.* 1990; Tannich *et al.* 1991); they also appear to contradict the proposal of Montfort *et al.* (1993) that amebal proteinases are not involved in pathogenicity which they base on changes of up to four times in total proteolytic activity, whereas we have shown here an increase of around 20 times using a CP-specific assay. It would be interesting to know the relation between our 75K CP and the 70K membrane-bound protease described recently by Ávila and Calderón (1993).

We have shown before that HM1 lysates produce an immediate fall in PD, Isc, and *R* in rabbit colon preparations (Navarro-García *et al.* 1993a), whose magnitudes are dose-dependent and correlate with the loss of intercellular junctions and the severity of the microscopic lesions observed in the mucosal cells (Navarro-García *et al.* 1993a,b). Using similar *in vitro* preparations, in this work we found that preincubation of 1659 lysates with either nonimmune or immune human serum prevented the fall in PD, Isc, and *R* (Fig. 5). These data suggest that a nonimmune component of serum inhibits the fall of electrical resistance, perhaps by preventing intercellular separation and damage to the epithelial cells caused by amebal lysates; these effects may be related to the "cytotoxicity" (McGowan *et al.* 1982; Lushbaugh *et al.* 1985) and proteolytic activity (Pérez-Montfort *et al.* 1987) known to be inhibited by nonimmune serum.

Our data suggest that CPs are indeed associated to *E. histolytica* pathogenicity and clearly indicate that amebae selected by their increased virulence through serial liver passage are also more virulent to the intestinal mucosa. On the other hand, the strategy used here to analyze *E. histolytica* pathogenicity, i.e., to compare trophozoites of increased virulence with their attenuated progenitors, appears to be more adequate than the opposite approach that compares attenuated variants, selected *a priori* by their functionally defficient phenotypes, with their more virulent progenitors.

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CAPITULO V

Eosinophil infiltration, homocytotropic IgE and type I hypersensitivity in the coecal mucosa of rats immunized intragastrically with *Entamoeba histolytica* trophozoites

Eosinophil infiltration, homocytotropic IgE and type I hypersensitivity in the coecal mucosa of rats immunized intragastrically with *Entamoeba histolytica* trophozoites

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Running title: Intestinal immunity to *Entamoeba histolytica*

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SUMMARY

No evidence had been published on the IgE role in the local immunity of intestinal amoebiasis, a parasitic infection known to induce specific coproantibody secretion in humans, antibody forming cells in Peyer's patches (PP) of mice, and IgA antibodies in the bile of rats, human milk and colostrum. We show here that intragastric immunization of rats with glutaraldehyde-fixed Entamoeba histolytica *trophozoites significantly increased the proportion of antiamoeba antibody-producing cells in the PP and spleen. The lamina propria of the coecum from immunized animals was infiltrated by eosinophils containing homocytotropic IgE antibodies. Antigenic challenge with amoebal lysates provoked an increase in the short-circuit current and the transepithelial potential difference in Ussing-chambered coecum preparations from immunized rats. These results indicate that type I hypersensitivity occurs in the coecum of intragastrically immunized rats, and suggest that eosinophils and IgE are involved in the local immunity and pathogenesis of intestinal amoebiasis.*

Keywords *Intestinal amoebiasis, intragastric immunization, antibody-producing cells, eosinophils, homocytotropic IgE, Ussing chambers, short-circuit current, potential difference, type I hypersensitivity, mucosal immunity*

INTRODUCTION

Amoebiasis, cosmopolitan infection produced by trophozoites of the protozoan parasite *Entamoeba histolytica*, is one of the most common parasitic diseases (Treviño *et al.* 1994). Approximately 400 million people worlwide are infected and some 30,000 die every year (Walsh 1986). In Mexico, about half a million cases of amoebiasis are registered per year only in the Mexican Social Security Institute (Treviño *et al.* 1994). Parasitic invasion of the mucosal layer of the large intestine depends on the invasiveness of each amoebal strain and the specific local immune response of the host (Navarro-García *et al.* 1994). Local immunity in intestinal amoebiasis has been poorly studied since most of the research has been focused on the systemic immunity (Kretschmer 1988).

To obtain protective immunity against intestinal amoebiasis it would be neccesary to induce local effector immune responses to *E. histolytica* trophozoites. Existence of these responses in human and experimental amoebiasis has been suggested by the presence of human coproantibodies (Sharma *et al.* 1981), antibody producing cells in mouse Peyer's patches (Moreno-Fierros *et al.* 1994), as well as secretory IgA in rat bile (Acosta *et al.* 1983) and human milk (Grundy *et al.* 1983) and colostrum (Acosta *et al.* 1985, López-Revilla *et al.* 1992a). Although mucosal immune responses are known to involve mainly the IgA and IgE isotypes (Jonard *et al.* 1984), IgE has not been shown to be involved in the local immunity to *E. histolytica*, as in other intestinal parasitic infections.

In this work we found a significant increase in the number of antiamoeba antibody producing cells (APC) in the Peyer's patches (PP) and the spleen of rats intragastrically immunized with glutaraldehyde-fixed *E. histolytica* trophozoites. We also observed local increase of IgE and eosinophil infiltration in the large intestinal mucosa. Furthermore, *in vitro* antigenic challenge with amoebal lysates provoked an immediate increase in the potential difference and short-circuit current values of preparations obtained from the coecum of immunized rats mounted in Ussing chambers, indicating that type I hypersensitivity does occur at least in experimental amoebiasis.

MATERIALS AND METHODS

Amoebal antigens

Fixed trophozoites: Immunizations were performed with *Entamoeba histolytica* HM1 trophozoites cultured in TYI-S-33 medium (Diamond *et al.* 1978) washed by centrifugation in cold TYI medium. The trophozoites were fixed with 0.25% glutaraldehyde, washed by centrifugation with 0.015 M phosphate buffer/0.15 M NaCl pH 7.4 (PBS), adjusted to 2×10^6 per ml. and stored at 4°C (López-Revilla *et al.* 1992a).

Proteins for ELISPOT: Recently harvested trophozoites were washed three times by centrifugation in cold TYI medium. The pellets were resuspended in 0.1 mM p-hydroxymercuribenzoate (pHMB) dissolved in 0.15 M Trizma-base (Sigma Chemical Co., St. Louis, MO, USA); total extracts were obtained by freezing in dry ice-acetone and thawing in boiling water (Moreno-Fierros *et al.* 1994). Before its use in ELISPOT assays, the protein patterns from amoebal extracts were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (López-Revilla *et al.* 1992b).

Amoebal lysates: The lysates used in the electrophysiologic experiments were obtained from trophozoites washed by centrifugation in cold TYI medium and stored in liquid nitrogen as minute pellets containing 5×10^5 trophozoites. At the beginning of each experiment, the pellets were suspended with 0.5 ml of Ringer's solution (López-Revilla *et al.* 1992a) and the amoebal lysates, obtained through a freeze-thawing cycle, were mixed with 2 ml of the same diluent at 37°C to obtain suspensions containing 20,000 lysed trophozoites per ml (Navarro-García *et al.* 1993a).

Inmunisations

Sprague Dawley male rats of approximately 200 g were treated with 40 mg of metronidazole (Flagyl®) administered by the intragastric route through a gauge 18 animal feeding needle for three consecutive days .

Immunizations started 10 days after the end of metronidazole treatment. Three groups of rats were used: *group 1*, treated with one dose of 2×10^6 *E. histolytica* HM1 trophozoites that

had been fixed with glutaraldehyde and suspended in 0.5 ml of magnesium aluminum hydroxide gel suspension; *group 2*, treated by the same route with four weekly doses of fixed trophozoites; and *group 3*, control, receiving only the vehicle.

Detection of antiamoeba antibody producing cells

Cell suspensions from Peyer's patches (PP) and spleen: From each rat the spleen was removed and 6-8 PP were dissected and placed in 15 ml Falcon tubes containing 5 ml of RPMI medium with 50 μg/ml gentamicin (Gibco, Long Island, NY, USA). The tissues were mechanically disaggregated by teasing through a steel mesh and filtering through a gauze, and centrifuged 10 min at 3000 rpm and 4°C. Cell pellets from PP were resuspended in 3 ml of RPMI medium with 10% fetal bovine serum (Hyclone, Road Logan, Utah, USA) whereas spleen cell pellets were resuspended in 10 ml of the same medium. Cell viability was determined by Trypan blue exclusion (Moreno-Fierros *et al.* 1994).

ELISPOT assay: Antiamoeba antibody producing cells (APC) were detected with a modified ELISPOT assay (Moreno-Fierros *et al.* 1994). Briefly, nitrocellulose membranes placed in 24-well culture plates were coated with 500 μl of PBS containing 100 μg of amoebal proteins and then blocked for 1 h with 6% defatted milk in 0.05% PBS-Tween. Five-hundred μl of spleen or PP cell suspensions with 2×10^5 cells/ml in RPMI medium with 10% foetal bovine serum were added to each well and the plates were incubated 4 h at 37°C in a humid atmosphere with 5% CO_2. To each well 500 μl of peroxidase-labelled polyclonal antibodies to rat immunoglobulin (HyClone) diluted 1:2000 were added and incubation proceeded for 1 h. Finally, 500 μl of "substrate solution" containing 3,3'-diaminobenzidine-nickel chloride-cobalt chloride and 0.005% H_2O_2 were added to each well. The nitrocellulose membranes were washed three times with 0.05% PBS-Tween between steps. The spots formed by APC were counted under a stereomicroscope at low magnification. The data were expressed as the mean ± standard deviation (SD) of the number of APC per 10^6 cells found in three membranes per rat, and the responses from the different groups of animals were compared using Student's *t* test.

Fixation and staining of coecum preparations

Rat coecum segments 2-3 cm long were rinsed clean of luminal contents with PBS and then fixed in paraformaldehyde-lysine-peryodate (McLean and Nakane 1974), embedded in paraffin, and cut into 4-6 μm sections that were stained either with haematoxylin and eosin (H&E), the Ziehl Neelsen metachromatic stain for mast cells (Atkins 1987) or the naphthalene black stain for eosinophilic leukocyte (Sanders 1974).

Immunohistochemistry

IgE detection in the coecal mucosa was performed immunohistochemically (Vega-López *et al.* 1992). Briefly, the sections were deparaffinised in xylene and endogenous peroxidase activity was quenched by rinsing with 1% H_2O_2 in methanol. The sections were washed three times with PBS and blocked with 2.5% normal goat serum for 30 min. A 50 μl-volume of mouse monoclonal antibodies to the rat IgE ε chain (MARE-1; Serotec, Kidlington, Oxford, England) diluted 1:500 was placed on the sections that were then incubated for 2 h at room temperature in a humid chamber. The sections were washed three times with PBS and incubated with 50 μl of biotinylated goat antimouse immunoglobulin for 1 h, followed by an additional 1 h incubation with streptavidin-horseradish peroxidase (Streptavidin-HRP system, goat anti-mouse IgG; Histomark, Gaithersburg, MD, USA). The reaction was visualized using 0.05% diaminobenzidine in 0.05 M Tris-HCl (pH 7.6) with 1% H_2O_2. The slides were counterstained with Harris haematoxylin, mounted in DPX (Aldrich Chemical, Milwaukee, WI, USA) and examined with a light microscope.

Electrophysiological measurements in coecal tissue

Coecal segments were removed from adult male rats under sodium pentobarbital anesthesia, placed in ice-cold Ringer's solution for mammals and gassed with an O_2/CO_2 (95%:5%) mixture. The excised segments were cut open along their mesenteric border, washed with cold Ringer's solution, divided into two fragments and mounted over the circular openings (6 mm diameter, 0.28 cm^2) of two adjacent Ussing hemichambers. Each hemichamber was filled with 2.5 ml of gassed Ringer's solution and kept at 37°C under constant O_2/CO_2 bubbling. Transmural potential

difference (PD) and short-circuit current (Isc) were recorded at 5-min intervals by means of a voltage clamp (López-Revilla *et al.* 1992, Navarro-García *et al.* 1993a).

Suspensions containing 50,000 lysed trophozoites in 2.5 ml Ringer's solution were added to the mucosal hemichamber of coecum preparations that had been mounted for 10 min; fresh Ringer's solution at 37°C was then added to the serosal hemichamber and both hemichambers were gassed with O_2/CO_2. Transmural resistance (R) values were obtained from PD and Isc values using Ohm's law equation (Navarro-García *et al.* 1993a, 1993b).

RESULTS

Increase of antiamoeba antibody producing cells

A single dose of trophozoites was enough to induce a substantial increase of antiamoeba APC in both PP and spleen (Fig. 1). The proportion of APC in PP was $(35 \pm 8.6) \times 10^{-6}$, around two times and significantly higher ($P < 0.05$) than in the spleen, whose corresponding value was $(18 \pm 6.9) \times 10^{-6}$.

In rats immunized with four doses of trophozoites the proportion of antibody producing cells increased significantly in the spleen ($P < 0.05$) but not in PP (Fig. 1).

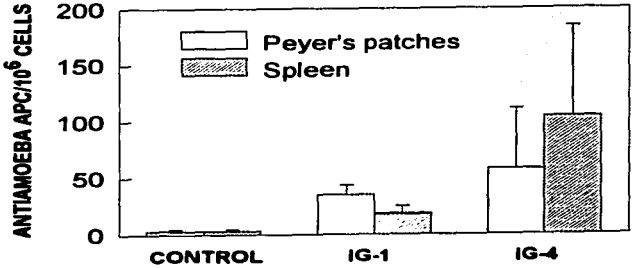

Figure 1. Antibody producing cells (APC) in Peyer's patches (PP) and spleens from rats intragastrically immunised with amoebae. Rats were immunized weekly with 2×10^6 glutaraldehyde-fixed *E. histolytica* HM1 trophozoites. APC were counted by an ELISPOT assay in samples obtained either one week after the first (**IG-1**) or after the fourth (**IG-4**) dose. Bars indicate the proportion of APC (mean ± SD) in PP and spleen cells from groups of three rats.

Eosinophils and IgE in the coecal mucosa

In sections of coecum samples from rats inmunised four times, a cellular infiltrate composed mainly of eosinophils was observed in the lamina propria as well as intraepithelially and in the submucosa (Fig. 2b). This infiltrate was discrete in the coecal mucosa of rats immunized with one dose and contrasted markedly with the slight infiltration observed in the coecal mucosa of nonimmunized rats (Fig. 2a). Tissue sections processed with the Ziehl Neelsen metachromatic stain showed no mast cell infiltration (data not shown), whereas, those stained with naphthalene black showed eosinophilic infiltration (Fig 2c).

Figure 2. Eosinophilic infiltration in the coecal mucosa of rats intragastrically immunized with four doses of fixed trophozoites. Normal (**a**) and immunized (**b** and **c**) rats. The animals were immunized with four weekly doses of glutaraldehyde-fixed trophozoites; coecal samples shown in **a** and **b** were fixed with paraformaldehyde-lysine-peryodate, embedded in paraffin and their sections stained with H&E, whereas the section shown in **c** was stained with naphtalene black.

Coecum sections from immunized animals incubated with monoclonal antibodies to rat ε chains showed a striking increase in the amount of mucosal IgE that was located mainly on eosinophils with classic bilobular nuclei (Fig. 3b, arrows) and on some mast cells with round nuclei (Fig. 3b, arrowhead). This finding contrasted with the normal infiltrate of such cells observed in nonimmunized rat samples (Fig. 3a).

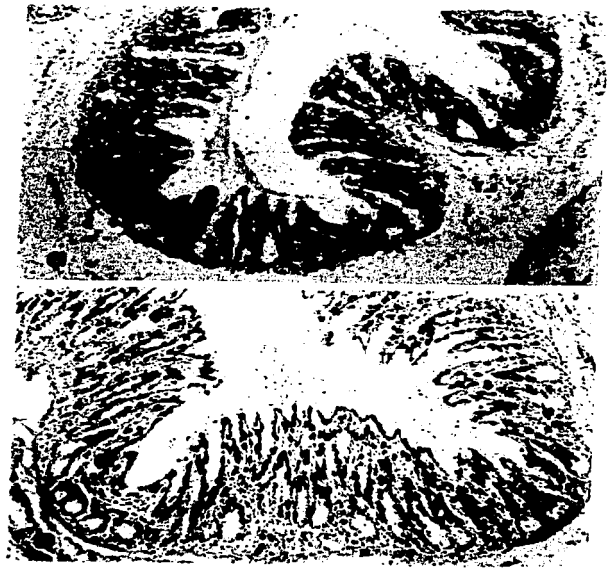

Figure 3. Increase of IgE in the coecal mucosa of rats intragastrically immunized with four doses of fixed trophozoites. Normal (**a**) and immunized (**b**) rats. Sections were obtained as in Fig. 2a, were stained with immunoperoxidase-diaminobenzidine after incubation with anti-ε monoclonal antibodies, and then counterstained with hematoxylin.

Increase of Isc and PD in immunized rat coecum by *in vitro* challenge

Coecum preparations from nonimmunized rats (n = 5) had initial values of 34.2 ± 5.3 μA cm^{-2} for Isc and 2.5 ± 0.5 mV for PD, which gradually decreased spontaneously to reach nil values after about 100 min (Fig. 4, Control). The initial R values averaged 82 ± 10 Ω cm^2 and were stable during most of the time that the experiments lasted.

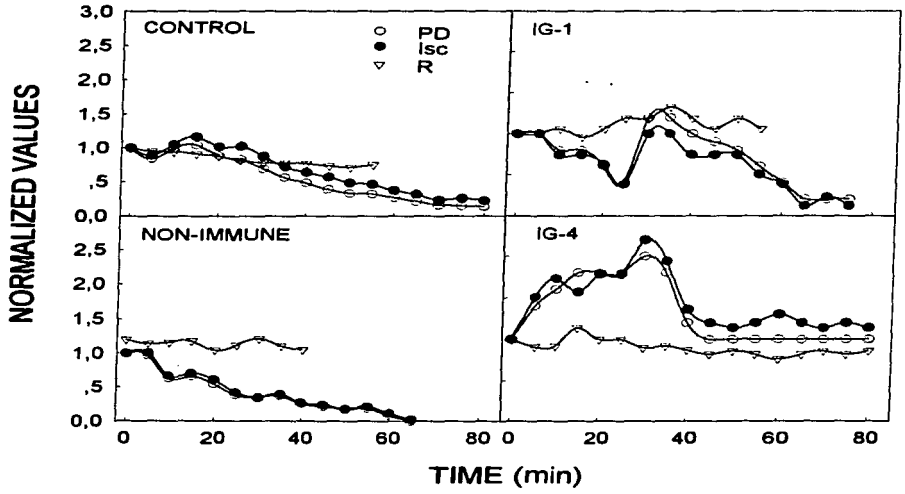

Fig. 4. Changes induced by *in vitro* challenge with amoebal lysates, of the short-circuit current (Isc) and the potential difference (PD) values of Ussing-chambered coecum preparations from immunized rats. (**CONTROL**) Untreated preparations from nonimmunized rats; (**NON IMMUNE**) preparations from nonimmunized rats, treated *in vitro* with amoebal lysates; preparations from rats that had been immunized with one dose (**IG-1**) or four doses (**IG-4**) of glutaraldehyde-fixed trophozoites and challenged *in vitro* with amoebal lysates. Initial values (mean ± SE) of the electrophysiological parameters were Isc_0 = 34.2 ± 5.3 μA cm^{-2}, PD_0 = 2.5 ± 0.5 mV and R_0 = 82 ± 10 Ω cm^2. The symbols represent normalized average values of the PD/PD_0, Isc/Isc_0 and R/R_0 ratios, i.e., (each time value)/(initial value); n = 4 for **CONTROL** and **NONIMMUNE**, n = 3 for **IG-1** and **IG-4**.

Preparations obtained from nonimmunized rats treated *in vitro* with amoebal lysates added to the luminal side of coecum segments showed an immediate decay of Isc and PD values. These were nil in about 60 min, as we had already found in rabbit colon and gerbil coecum preparations (Navarro-García *et al.* 1993a, 1995).

Preparations from rats killed one week either after the first (IG-1) or the fourth (IG-4) intragastric immunization were challenged *in vitro*. The challenge, done by adding amoebal lysates to the luminal side of coecum segments, provoked an immediate increase of the Isc and PD values, whose magnitudes depended on the number of immunizations (Fig. 4, IG-1 and IG-4) and correlated with the eosinophilic infiltration and the amount of IgE present in the coecal mucosa (see Figs. 2 and 3). Furthermore, coecum preparations from rats immunized four times maintained their electrophysiological properties for a much longer time that the corresponding control preparations.

DISCUSSION

Although *E. histolytica* is an intestinal parasite, little is known about the mucosal immune response it induces. To explore such response experimentally amoebal immunogens should administered through the oral or intragastric routes (Mestecky and McGhee 1989). The use of particulate antigens appeared to be convenient because, with the exception of cholera toxin, soluble antigens are much less effective than particulate antigens to induce intestinal immunity (Biewenga *et al.* 1993). In this work we found that intragastric immunization of rats with glutaraldehyde-fixed trophozoites (GTF) induced a substantial antibody-producing cell response in PP and spleen, as has recently been reported for mice in which GTF were administered by the rectal and intragastric routes (Moreno-Fierros *et al.* 1995). In rat spleen the response increased with the number of doses, whereas in PP it was dose-independent. The use of an ELISPOT assay allowed us to detect homocytotropic IgE containing cells whose increase could not have been predicted if we had searched for antiamoeba IgE antibodies in the serum or in intestinal secretions.

Immune mucosal efector cells and molecules along the intestine are found mainly in the lamina propria (Biewenga *et al.* 1993), where antibody responses involve mainly IgA and IgE isotypes (Jonard *et al.* 1984). Coecum samples from rats intragastrically immunized by us with one or four doses of trophozoites showed significant increases in the levels of IgA (not shown) as well as of IgE in the lamina propria. Furthermore, histochemically the levels of IgE in the intestinal mucosa appeared to be as high as those of IgA. Homocytotropic IgE appeared to be associated mainly with eosinophils and some mast cell but was not detected free, as the secretory IgA (sIgA) found in the lamina propria of immunized rats (Brandtzaeg 1983). These findings suggested that eosinophils and mast cells might have increased in the coecal mucosa of immunized rats. Coecum sections stained with naphthalene black showed eosinophilic infiltration in the lamina propria as well as intraepithelially and ocasionally in the submucosa, whereas sections stained with the Ziehl Neelsen technique showed no mast cell infiltration.

The eosinophil has been recognized as an important effector cell in protozoan infections caused by *Trypanosoma cruzi* and *Isospora belli* (Sanderson et al. 1977, Weller 1991). Some eosinophils are regularly found in the early inflammatory reaction to invading amoebae in experimental liver amoebiasis (Tsutsumi & Martínez-Palomo 1988). López-Osuna *et al.* (1992) have shown that only activated eosinophils destroy *E. histolytica* trophozoites *in vitro*.

It is well known that IgE antibodies play an important role in the local immunity to intestinal nematode parasites (Castro 1982). On the other hand, mucosal tissues are rich in mast cells, which may be sensitised by the IgE antibodies formed in the mucosal lymphoid structures and play a substantial role in type I hypersensitivity reactions (Baird *et al.* 1985; Russell 1986). Other cells expressing IgE receptors, such as eosinophils, might also be involved in the immune protective mechanisms of hosts infected by intestinal parasites.

We show here that the *in vitro* challenge of coecum preparations from GFT-immunized rats with amoebal lysates caused an immediate increase in their Isc and PD. The electrophysiologic response was higher in rats immunized four times, indicating the occurrence of type I hypersensitivity in our experimental model. Another possible role for IgE antibodies is to increase vascular permeability, resulting in an increase in the local concentration of antibodies of other isotypes (Steinberg *et al.* 1974). On the other hand, the finding that coecum preparations

from rats immunized and challenged *in vitro* maintain their electrophysiological properties much longer than non-immune preparations, suggests that an antiamoebic protective effect mediated by local immunity is induced by intragastric immunization with GFT. This effect resembles that observed in the intestine of *Schistosoma mansoni* infected animals reflecting an immediate hypersensitivity reaction combined with the subsequent IgE mediated effector response involving eosinophils, macrophages and platelets attracted to the site of penetration by parasite antigens that induce mast cell degranulation (Hagan 1993). Abu-Ghazaleh *et al.* (1989) have found that eosinophil degranulation induced by sIgA may be the major Ig-mediated effector function at mucosal surfaces in helminth infections and hypersensitivity diseases.

Since ion transport is stimulated when the intestinal mucosa of infected rats is challenged *in vitro* with crude antigenic extracts of *Nippostrongylus brasiliensis* (Baird *et al.* 1985), *Trichinella spiralis* (Castro *et al.* 1987) or *Fasciola hepatica* (O'Malley *et al.* 1993), the increase in Isc and PD values in our model could be due to antigen-stimulated anion secretion, cation absorption or both (O'Malley *et al.* 1993) in the presence of homocytotropic IgE antibodies mediating an anaphylactic reaction in the rat coecum mucosa.

Local IgE production by lamina propria plasma cells may be the source of homocytotropic IgE in the rats intragastrically immunized with GFT, because the increase of serum IgE is not clear. Geller *et al.* (1978) have reported normal serum IgE levels in human intestinal amoebic infection, while Dasgupta (1974) claim to have found high levels in both invasive and luminal amoebiasis.

Amoebal antigens could react with local homocytotropic IgE to provoke degranulation of intestinal cells (O'Malley *et al.* 1993) and the subsequent liberation of pharmacologically active substances such as histamine, prostaglandins, and leukotrienes (Castro 1982, Castro *et al.* 1987). These antibodies could also play a role in the early inflammatory reaction of invasive intestinal amoebiasis, with participation of eosinophils, Charcot-Leyden crystals and mast cells (Juniper *et al.* 1958). Some symptoms of amoebic dysentery (colic, abdominal tension, tenesm and bloody stools) may be due to intestinal anaphylaxis, as first suggested by Kretschmer (1984).

The immediate skin hypersensitivity reaction to crude *E. histolytica* antigen observed in human amoebiasis (Kretschmer *et al.* 1972), led Kretschmer (1984) to suggest that mast cells and

IgE might be involved in this reaction. In our experimental model eosinophils and specific IgE appear be involved in type I hypersensitivity, and therefore their role may be explored in experimental intestinal amoebiasis.

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CAPITULO VI

Conclusiones y Perspectivas

CONCLUSIONES

El desafío con lisados amibianos a preparaciones intestinales provenientes de animales normales o previamente inmunizados nos ha permitido analizar los mecanismos patogénicos e inmunológicos involucrados en la amibiasis intestinal. Estas evidencias son presentadas en los capítulos III, IV y V de esta tesis.

A continuación enumero las conclusiones más importantes:

CAPITULO III

1) La exposición a los lisados por más de 80 min produjo daños al colon dependientes de la dosis y del tiempo de exposición y manifestados por:

a) incremento de la velocidad de caída de la diferencia de potencial, corriente de corto circuito y resistencia transmural

b) daños a la mucosa que van desde vacuolización subnuclear y acortamiento de las células epiteliales hasta la pérdida de uniones intercelulares, destrucción de las microvellosidades, y necrosis de zonas epiteliales interglandulares.

2) Las lesiones estructurales mostradas en este trabajo se asemejan a la serie de lesiones descritas en en biopsias humanas (Prathap y Gilman, 1970), en modelos animales (Takeuchi y Phillips, 1975) y en células en cultivo (Knight et al. 1974, Orozco et al. 1982)

3) Nuestro modelo tiene además las siguientes ventajas sobre otros modelos *in vitro* y en animales enteros:

a) es más cercano a la amibiasis intestinal porque el blanco es un segmento intestinal y no células en cultivo.

b) las propiedades electrofisiológicas de los segmentos expuestos a los trofozoitos lisados pueden correlacionarse con los cambios morfológicos y bioquímicos.

c) la localización de las lesiones microscópicas es sencilla porque la interacción intestino-lisados ocurre en un área mínima y definida.

d) los resultados son inmediatos, reproducibles y cuantificables, en contraste con los de

animales inoculados intracecalmente, que son inconsistentes y muy tardados.

CAPITULO IV

4) Una contribución interesante de esta parte del trabajo es que la comparación de las propiedades de los trofozoítos de virulencia aumentada con sus progenitores atenuados parece ser una estrategia más adecuada que la usada habitualmente, que consiste en comparar la virulencia de variantes amibianas atenuadas —seleccionadas a priori por su fenotipo deficiente en funciones celulares que podrían no tener relación directa con la virulencia— con la de las cepas parentales de las que provienen.

5) Con esta estrategia inoculamos trofozoítos de la cepa HM1 (que había sido mantenida en el laboratorio por más de 15 años) en hígado de hamster; a través de cuatro pases sucesivos obtuvimos la clona 1659 de alta virulencia. La comparación de la actividad enterotóxica *in vitro*, de las cisteín-proteasas (CP) y de las gelatinasas de los lisados amibianos de ambas cepas nos permitió llegar a las siguentes conclusiones:

a) El tratamiento de segmentos de ciego de jerbo con lisados amibianos de la clona 1659 causa una caída más rápida de las propiedades electrofisiológicas (DP, Icc y R) del ciego de jerbo que con la cepa parental atenuada HM1.

b) El suero humano normal e inmune (provenientes de personas con absceso hepático amibiano) y el E-64 (inhibidor específico de CP) pevienen la caída de las propiedades electrofisiológicas.

c) Las gelatinasas, menos activas en trofozoítos HM1 que en 1659, fueron mejor preservadas en lisados que contenían pHMB 10 mM para prevenir la autoproteólisis. En lisados HM1 la actividad proteolítica principal se debe tal vez a la gelatinasa de 53 kDa, localizada principalmente en la pastilla y por tanto asociada a componentes subcelulares no disueltos (membranas?). En la clona 1659, de mayor virulencia, la actividad proteolítica aumenta aparentemente por el aumento de las gelatinasas de 53 y 75 kDa, aunque el mayor aumento relativo corresponde a la de 75 kDa, localizada principalmente en la fracción sobrenadante y prácticamente ausente en HM1.

d) La actividad total de CP fue 17 veces más alta en lisados 1659 que en HM1, fue

inhibida con E-64 (dosis inhibitoria media 20 μM), fue estimulada por 2-mercaptoetanol (ME) y reactivada por ME en lisados que contenian pHMB.

6) Estos hallazgos indican que el incremento de la virulencia de *E. histolytica* correlaciona con un notable aumento de la actividad enterotóxica *in vitro* y de dos CPs (53 y 75 kDa), sugiriendo que las proteasas son un factor significativo de patogenicidad.

CAPITULO V

Como los daños electrofisiológicos y morfológicos a las células epiteliales de la mucosa intestinal por los lisados amibianos pudieran estar influenciados por la inmunidad local, realizamos experimentos de reto antigénico (con lisados amibianos) en preparaciones de intestino de animales que habían sido inmunizados con trofozoítos fijados con glutaraldehido. Esta estrategia inmunofisiológica nos permitió obtener las siguientes conclusiones

7) La inmunización de ratas por la vía intragástrica con amibas fijadas con glutaraldehido:

a) Incrementó las células productoras de anticuerpos antiamibianos en las placas de Peyer y el bazo

b) La lámina propia del ciego de animales inmunizados tuvo un infiltrado principalmente de eosinófilos que contenían anticuerpos homocitotrópicos de la clase IgE.

c) El reto antigénico (con lisados amibianos) provocó un incremento en los valores de Icc y DP de preparaciones de ciego montadas en cámaras de Ussing.

8) Estos resultados indican que ocurre hipesensibilidad de tipo I en el ciego de ratas inmunizadas intragástricamente, y sugiere que los eosinófilos y la IgE pueden ser factores significativos en la inmunidad y por tanto en la patogénesis de la amibiasis intestinal.

PERSPECTIVAS

Como los factores determinantes del curso de la infección amibiana son la invasividad del parásito y la inmunidad del hospedero, actualmente estamos realizando —en colaboración con la doctora Miriam Pedroso, inmunóloga del Centro Nacional de Sanidad Agropecuaria de La Habana— experimentos electrofisiológicos en los que desafiamos con lisados amibianos a

preparaciones de intestino de animales inmunizados con trofozoítos solos o acompañados con la toxina del cólera (el mejor adyuvante conocido por vía oral) y un inmunoestimulante (ß-1,3 glucano derivado de la pared de levaduras), producido en Cuba. Estos experimentos son paralelos a otros que estamos llevando a cabo para caracterizar la inmunidad antiamibiana intestinal y sistémica, tanto en su rama inductora (formación de células productoras de anticuerpos en las placas de Peyer y el bazo) como en la efectora (anticuerpos en el suero, en el contenido intestinal y en las heces, así como anticuerpos, interleucinas y linfocitos T y B en la lámina propia del intestino grueso).

Como hemos mostrado en este trabajo, las proteasas amibianas y en especial las cisteín-proteasas juegan un papel importante en la virulencia amibiana. La purificación de estas proteasas permitirá producir anticuerpos monoclonales contra ellas, identificar cuáles se incrementan al aumentar la virulencia, y clonar los genes correspondientes. Los anticuerpos monoclonales y los genes clonados podrían servir para determinar la participación de las CP en la invasividad, desarrollar nuevos métodos de diagnóstico y quizás producir antígenos relevantes para inmunizar contra la amibiasis.

Usando la electrofisiología estamos haciendo experimentos para determinar la participación de la inmunidad local en la amibiasis y en otras infecciones de las mucosas. Gilbert Castro, padre de la inmunofisiología, propuso que sería ventajoso e incluso necesario estudiar a los párasitos intestinales para analizar la respuesta del hospedero desde la perspectiva inmunofisiológica y reconciliar fenómenos o eventos que no pueden ser explicados por los conceptos o técnicas disciplinarias tradicionales. Pueden crearse modelos de secreción intestinal mediada por células cebadas mediante la adición de antiinmunoglobulina E a la solución serosa del colon de rata en cámaras de Ussing. Puede disecarse el papel de los diferentes mediadores inflamatorios o del sistema nervioso entérico mediante el pretratamiento del tejido intestinal con antagonistas específicos (por ejemplo antihistamínicos) o inhibidores de la síntesis de los mediadores químicos (por ejemplo bloqueadores de la cicloxigenasa) en las cámaras de Ussing antes de la estimulación, o mediante la adición de inhibidores de los neurotransmisores.

Por último, uno de mis intereses es el desarrollo de vacunas. La prevención de muchas infecciones de las mucosas podrá realizarse de manera más conveniente con vacunas orales polivalentes, las vacunas del futuro. El desarrollo de estas vacunas depende de los avances en el conocimiento de los componentes y mecanismos de la fisiología e inmunidad de las mucosas, en el conocimiento de los mecanismos de la virulencia de los patógenos adherentes y la identificación y clonación de los genes correspondientes y, finalmente, en la producción de los antígenos relevantes con técnicas de DNA recombinanteo síntesis química. Como las vacunas orales contra las enfermedades infecciosas de los países pobres no interesan a los países desarrollados, nosotros debemos desarrollarlas.