la respuesta mitogénica de linfocitos de sangre periférica de pacientes con amibiasis estaba disminuida durante la primera semana de convalecencia luego aumentaba y finalmente tendía a disminuir, tres semanas después de iniciada la enfermedad.

En otro estudio realizado con pacientes convalecientes de AHA y controles sanos se analizó la respuesta mitogénica de linfocitos humanos a antígeno amibiano sometido a varios ciclos de congelación/descongelación y sonicación: No se observaron diferencias entre la mitosis inducida en linfocitos de individuos sanos en presencia o ausencia de antígeno amibiano (Sabanat, 1973). Además, la respuesta de los linfocitos a la fitohemaglutinina fue superior a la inducida por el antígeno amibiano.

Es importante comentar con respecto al presente estudio, que la persona de la que se obtuvieron las células mononucleares periféricas para realizar los ensayos de mitosis nunca había padecido de AHA, pero sí colitis amibiana. En este caso sus linfocitos presentaron una respuesta mitótica a nuestra preparación de antígeno similar a la producida por la fitohemaglutinina, cosa que no sucedió en los estudios arriba comentados; lo cual sugiere fuertemente que la respuesta mitogénica in *vitro* de las CMP depende en gran medida de la calidad del antígeno, además del posible efecto inhibitorio producido por *E. histolytica* en los pacientes con AHA, arriba mencionado. Nosotros pensamos que obtuvimos una mejor respuesta mitogénica al ATA particulado porque protegimos a éste de la auto-degradación, pre-tratándolo con cloroformo-metanol (Said-Fernández, 1996, Tamez-Treviño y cols., 2000). Este procedimiento es mucho más efectivo y seguro

que la utilización de inhibidores de proteasas, y no había propuesto previamente para realizar este tipo de ensayos.

Como parte de nuestra estrategia, utilizamos antígeno total de trofozoítos, a diferencia de otros grupos, que utilizan membranas plasmáticas aisladas (Sabanat, 1973 y Ganguly, 1981). Como antes comentamos, la ventaja de utilizar la totalidad de los antígenos amibianos es la de no descartar *a priori* antígenos capaces de inducir una respuesta protectora eficaz. Aunque era posible que en la mezcla de antígenos presentes en el ATA podría haber algunos que estimularan la respuesta inmune y que otros la inhibieran. Decidimos iniciar nuestro proyecto de investigación con antígeno total amibiano pensando que si no descubríamos una respuesta mitogénica HPLC podríamos fraccionar este antígeno y probar las fracciones por separado, pero esto no fue necesario.

Nos llamó particularmente la atención que el mejor tiempo de incubación, después de haber puesto en contacto a las CMP con el ATA y de agregar a las mezclas de ensayo la [^{3}H]-TdR (1 µCi/10 µl) fue de 12 h (Fig. 3), mientras que otros autores han encontrado que 18 h es el tiempo óptimo. Esto puede explicarse porque después de los 3 y medio días de cultivo y las células ya pasado por una expansión clonal y han entrado en apoptosis (Janeway y Travers, 1997).

Al igual otro estudio (Ganguly, 1981) donde se buscó proliferación de linfocitos de sangre periférica de pacientes convalecientes de AHA utilizando antígenos amibianos, nosotros encontramos una respuesta similar a la observada en los controles sanos, esta respuesta estaba inhibida durante la primera semana de la convalecencia luego aumentaba y finalmente tendía a disminuir a las tres

semanas después de la enfermedad. Como esperábamos las máxima respuesta mitogénica fue inducida por el ATA particulado (Datos no mostrados). Sin embargo en estudios previos se ha utilizado antígeno soluble con buena respuesta (Salata, 1982).

El ATA soluble fue capaz de estimular la producción de NO en un cultivo de CMP humanas (Fig. 5-6). Probamos también el ATA particulado, pero los resultados preliminares fueron demasiado dispersos (datos no mostrados). Esto porque los ensayos realizados con ATA particulado eran demasiado turbios y el método de Greiss es colorimétrico. Entonces decidimos continuar el presente trabajo con ATA soluble.

Cuando iniciamos el presente proyecto algunos autores afirmaban que las CMP no eran capaces de producir *in vitro* este compuesto. Nuestros resultados muestran que si; y otros autores también lo han hecho, utilizando diversos estímulos (Bogdan, 2000, Weinberg, 1998 y Kroncke, 1998, Murray, 1992, Nosaki, 1997). Entonces, ahora está muy HPLC o que los macrófagos humanos son capaces de producir NO *in vitro* cuando son estimuladas por un antígeno adecuado y en una forma adecuada. Por otro lado, en los trabajos referentes a la producción de NO que se han realizado previamente para identificar antígenos de amibas capaces de inducir una respuesta protectora se habían utilizando cultivos de macrófagos murinos (Denis y Chadee, 1989, Seguin, 1995 y 1997). Así por ejemplo, se había demostrado que la lectina de trofozoítos de *E. histolytica* que se une a galactosa (*gal*-lectin) es capaz de estimularla producción de NO en cultivos macrófagos derivados de médula ósea de ratón (Seguin, 1995; 1997).

La producción de NO es dependiente de citocinas, principalmente INF-γ y TNF-α (Seguin, 1997; Janeway y Travers, 1997). La producción de NO depende de la actividad de la enzima iNOS sintasa y que puede ser bloqueada por falta de sustrato (L-arginina) (Lin, 1992.) o por bloqueo de la actividad enzimática con inhibidores y/o análogos del sustrato (Lin, 1994). Nuestros resultados son acordes con estas afirmaciones, pero utilizando CMP humanas (Fig. 5-6), y también indican que la producción de NO por CMP humanas es dependiente del procesamiento y presentación del antígeno amibiano, pues la producción de NO pudo ser inhibida significativamente al detener el proceso de presentación del antígeno, incubando las células con ATA y después fijándolas con glutaraldehído 0.05% por diferentes tiempos: a mayor tiempo disponible para la presentación del antígeno (antes de ser fijadas con glutaraldehído), mayor producción de NO (Fig 9).

Estudios previos se han demostrado que la producción de NO por macrófagos murinos esta correlacionada con la actividad amebicida de los mismos (Denis y Chadee ,1989; Lin , 1994; Seguin , 1995;1997). Por ello se considera que el NO es la principal molécula efectora que utilizan los macrófagos para matar a las amibas (Lin , 1992). Como antes comentamos, resultados previos de nuestro grupo apoyan este postulado, porque el MAIF, producido por células de linfoma murino de la línea L517810 es capaz de inhibir la producción *in vitro* de NO (González-Garza y cols., 2000), y de permitir el desarrollo de AHA en ratones Balb/c inoculados experimentalmente, a pesar de que estos animales son naturalmente resistentes a la infección por *E. histolytica* (Salata, 1982). En el

presente trabajo nosotros demostramos que los macrófagos humanos estimulados por las fracciones 4 y 9 del ATA soluble, digeridos con tripsina y separadas por HPLC, son capaces de producir NO (Figs. 11-12) . La fracción que indujo la mayor producción de NO en CMP humanas fue la fracción 9, y la concentración que indujo la mayor respuesta fue la equivalente a 8.5 mg de proteínas/ ml (Fig. 12), mientras que estudios previos han reportado inducción de la producción de NO utilizando concentraciones de antígenos diversos 1-50 mg/ ml.

En este trabajo observamos que el ATA tiene la propiedad de co-estimular la producción de NO en CMP inducidas por LPS y/o interferón-γ (Fig. 7-8) . Lo que nos indica una respuesta dependiente de la activación de linfocitos T. Además, demostramos que estos macrófagos activados con las fracciones 4 y 9 del antígeno amibiano soluble separadas por HPLC son capaces de matar al 37% de las amibas cuando se co-cultivan con trofozoítos de *E. histolytica* (Fig 13). Para obtener fracciones del antígeno amibiano total que fueron capaces de inducir la producción de NO, digerimos el ATA soluble con tripsina, y ultrafiltramos la preparación con un corte de 5,000 kDa. Así, eliminamos las proteínas y otras moléculas de alto peso molecular y conservamos únicamente los péptidos más pequeños. Estos péptidos los separamos por HPLC, y obtuvimos cinco picos principales y otros 20 picos más pequeños (Fig 10). El método utilizado en la cromatografía fue de fase reversa, utilizando un gradiente de acetonitrilo y ácido trifluoroacético en una columna C18 (de 5 μm de tamaño de poro y 3.7 mm de diámetro interno y 200 mm de largo). Estas columnas separan moléculas según su peso molecular y su carga eléctrica total. Esta estrategia es la que se utiliza regularmente para separar

péptidos pequeños por HPLC. Cuando analizamos la capacidad de inducir producción de NO de cada una de las fracciones separadas por HPLC encontramos la mayor actividad inductora en dos de las fracciones separadas la fracción 4 y la 9 y que corresponden a 2 de los picos que se observan en la separación por HPLC P1 y P3. Sin embargo en ese momento no podíamos saber si el resultado de esta producción mayor era por efecto de concentración, debido a que casualmente estas fracciones no sólo mostraron el mayor efecto, sino también la mayor concentración de péptidos en los picos de separación correspondientes en el HPLC [(mayor A_{220}) (Fig. 10)]. Por ello estandarizamos las concentraciones de proteína en todas las fracciones separadas por HPLC y los resultados fueron similares a los iniciales. Es decir, las mismas dos fracciones la 4 y la 9 indujeron la mayor producción de NO (Fig 12). Rosanne Seguin y colaboradores, en 1997, así como Denis y Chadee en 1987, describieron la capacidad de los macrófagos activados con antígenos amibianos de matar a trofozoítos patógenos de *E. histolytica.* Nosotros encontramos que las células mononucleares periféricas obtenidas de sangre fresca total de un voluntario sano son capaces de disminuir la viabilidad de trofozoítos patógenos de amibas cepa HM1-IMSS, cuando son previamente activadas. Es decir, que de el ATA es capaz de activar a las CMP para matar a las amibas, como se demuestra en la figura 13. Esta actividad citotóxica de las CMP es dependiente casi totalmente de la actividad de los macrófagos, pues en este ensayo trabajamos únicamente con las células adheridas a las paredes de los pozos, mientras de que los sobrenadantes se desecharon. Como los macrófagos son las únicas células adherentes de las CMP, deducimos que la capacidad citotóxica es dependiente de la actividad de estos.

CAPITULO 5

CONCLUSION

5.1 CONCLUSIONES

1) El antígeno amibiano total ATA, presentado a las CMP en forma soluble o particulada, al cual se le eliminó la actividad de las proteasas endógenas mediante un tratamiento con cloroformo-metanol es capaz de inducir una respuesta inmune celular in *vitro*.

2) El ATA es capaz de inducir la proliferación de linfocitos en cultivos ce CMP con una eficacia similar a la observada con la fitohemaglutinina y mayor que la lograda hasta ahora por otros grupos.

3) El ATA induce la producción de NO en cultivos de macrófagos obtenidos de sangre periférica humana.

4) La inducción de la activación de macrófagos en cultivos de CMP, con producción de NO se incrementa por co-estimulación con interferón gama o LPS, lo cual indica que es una actividad dependiente de la activación de linfocitos T.

4) La inducción de la activación de macrófagos con producción de NO es dependiente de la presentación del antígeno.

5) Demostramos que es posible separar fracciones del antígeno total mediante HPLC.

6) Las fracciones del ATA digeridas con tripsina y separadas por HPLC son capaces de inducir la producción de NO, en presencia de LPS en los cultivos de macrófagos ya mencionados, con una eficacia 2.4 Veces mayor que el ATA total.

7) La tripsina incubada sola y autodigerida exactamente igual que el ATA con tripsina no es capaz por si misma de inducir producción de NO en los cultivos de macrófagos humanos, bajo condiciones experimentales exactamente iguales a las que sometimos al ATA o sus fracciones, por lo que concluimos que el efecto inductor de activación de macrófagos humanos con producción de NO es un efecto específico de antígenos amibianos y no de fracciones producto de la autodigestión de la tripsina.

8) Los macrófagos humanos de cultivos de CMP, activados con las fracciones 4 y 9 separadas por HPLC. No sólo indujeron la producción de NO in vitro, sino que fueron capaces de estimular a los macrófagos activados para destruir trofozoítos, invirtiendo la secuencia normal de hechos en co-cultivos de amibas y macrófagos, en los que las amibas destruyen preferentemente a los macrófagos no activados.

9) Tomando en cuenta todo lo anterior concluimos sobre nuestro trabajo completo que nuestros resultados sostienen la hipótesis que planteamos en el proyecto que originó el presente trabajo.

5.2 PERSPECTIVAS

Haber encontrado dos fracciones peptídicas capaces de inducir la producción de NO en macrófagos humanos abre amplias perspectivas para identificar al menos 2 antígenos amibianos que fueron aislados e identificados mediante una cuidadosa preservación de la integridad física del ATA. Estos antigenos no fueron preseleccionados considerando las funciones biológicas ni la

ubicación en el cuerpo celular de los trofozoítos de *E. histolytica* , sino que dejamos a la naturaleza que nos respondiera una pregunta que consideramos fundamental para el desarrollo de la vacuna contra la amibiasis, y que otros grupos no se habían planteado: ¿Que antígenos amibianos son capaces de inducir la activación de macrófagos humanos con producción de NO, y por lo tanto con verdaderas posibilidades de inducir una respuesta protectora contra la amibiasis en seres humanos?. Como respuesta a ello, nosotros localizamos 2 fracciones conteniendo péptidos de 10 kDa o menores capaces de inducir in vitro la activación de macrófagos en presencia de linfocitos de sangre periférica T $CD4^+$ y T $CD8^+$, pero aún no sabemos si su secuencia es homogénea. Es decir, si existen en estas fracciones varios péptidos con diferente secuencia, ni tampoco a que proteínas pertenecen éstos. Por lo tanto tampoco sabemos donde están ubicadas estas proteínas, ni cual es su función biológica. Sabemos, por otro lado, que estos péptidos deben ser presentados para activar la respuesta inmune celular, pero no conocemos la secuencia de los epitopos presentados por los macrófagos, ni si la respuesta inductora de producción de NO es dependiente de la actividad de iNOS sintasa y de la presencia de citocinas como interferón γ o TNF α. Es necesario investigar si los péptidos obtenidos en este trabajo u otros, separados mediante la estrategia seguida aquí son capaces de inducir una respuesta humoral adecuada, dado que sabemos ahora que ambas respuestas, la celular y la humoral, son importantes para inducir una respuesta protectora adecuada. Entonces deberá investigarse si los péptidos inductores de una respuesta celular *in vitro* contra *E. histolytica* y los inductores de una respuesta celular adecuada (por ejemplo

3.- Aislamos e identificamos dos fracciones de antígenos de *Entamoeba histolytica* que son capaces de inducir la producción de NO en cultivos de macrófagos humanos.

4.- Confirmamos la capacidad de los macrófagos humanos de producir NO *in vitro.*

5.- A nuestro juicio, abrimos un nuevo camino, con enfoque y estrategias muy novedosos, y que parece ser muy promisorio, considerando los fundamentos teóricos de nuestra estrategia y los resultados aquí mostrados, para el desarrollo de una vacuna contra la amibiasis.

CAPITULO 6

6.1 BIBLIOGRAFÍA

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V. Diseño de vacunas contra enfermedades infecciosas

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Resumen

Las vacunas son la forma más efectiva y económica de prevenir y controlar enfermedades infecciosas y parasitarias. A pesar del gran desarrollo que este tipo de biológicos ha experimentado en los últimos años, todavía hay enfermedades producidas por virus, como el SIDA y por protozoarios, como la amibiasis y la tripanosomiasis, que no han podido ser controladas mediante vacunas eficaces y seguras. La vacuna contra la malaria es una de las que mayor éxito ha tenido. Pero todas éstas aún están en pleno desarrollo. Existen numerosos factores que se oponen al éxito de las vacunas antes mencionadas. Algunos de ellos son el riesgo que se corre al utilizar virus vivos o DNA, en el caso del SIDA o de la capacidad que tienen los protozoarios para evadir, deprimir o inactivar la respuesta inmune protectora del huésped. Una vacuna efectiva debe inducir tanto la respuesta humoral como la respuesta celular del sistema inmune y considerar el ciclo biológico del agente causal. Se discuten aquí diversas y novedosas estrategias para desarrollar vacunas tanto contra SIDA como contra la amibiasis.

Palabras clave: *Vacunas, infecciones, protozoarios.*

Summary

Vaccines are the most effective and reliable way to prevent and control infectious and parasitic diseases. In spite of the great development that this kind of biological products has reached over the past years, certain viral diseases, like AIDS and most of those produced by parasitic protozoa, like amebiasis and trypanosomiasis, still have not been controlled by means of safe and effective vaccines. Vaccine against malaria is one that has shown greater success. Accordingly, all are still being developed. Numerous factors oppose success. For example, one is the risk that includes the use of live viruses or their DNA, in the case of HIV or the ability that some protozoa have to depress, evade, or inactivate the protective immune response. An effective vaccine must induce both the humoral and the cellular branches of the immune system, and in addition to considering the entire biological cycle of the causative agent. In this contribution, several novel strategies to develop vaccines against amebiasis and AIDS are discussed.

Key words: *Vaccines, parasitic diseases.*

Introducción

Una vacuna puede salvar más vidas y más dinero que cualquier otra intervención médica.[1]

Hasta hace relativamente poco tiempo el concepto de vacuna se aplicaba exclusivamente a la prevención de enfermedades infecciosas y fue hasta hace poco más de 30 años que empezaron a aparecer resultados prometedores sobre el desarrollo de vacunas dirigidas contra enfermedades causadas por parásitos, las cuales están aún en pleno desarrollo.

De acuerdo con Adrian J. Ivinson[1] existen tres conceptos sobre las vacunas modernas que deben considerarse con especial atención para lograr los efectos benéficos deseados: (a) aspectos biológicas, (b) su costo/beneficio y (c) la cooperación internacional para aplicarlas en la mayor cantidad posible de personas en las áreas del mundo donde se les requiere.

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Nosotros dedicaremos esta contribución a un tema relacionado con las características biológicas de las vacunas: su diseño, desarrollo y resultados, sin pretender que ésta sea una revisión exhaustiva. Nuestro objetivo es sólo presentar algunos ejemplos sobre las estrategias más sobresalientes que se están abordando para desarrollar vacunas contra dos enfermedades importantes por su incidencia y mortalidad y que por las características biológicas de sus agentes causales ha sido extraordinariamente difícil desarrollar productos que protejan en forma segura, efectiva y duradera. Estas dos enfermedades son el Síndrome de la Inmunodeficiencia Adquirida (SIDA), causada por el virus de la inmunodeficiencia humana (VIH), uno de los retrovirus más complejos conocidos hasta ahora; y la Amibiasis, cuyo agente causal es el protozoario *Entamoeba histolytica*. El desarrollo de las vacunas contra el SIDA y enfermedades causadas por protozoarios está planteando a los investigadores difíciles y variados problemas, que como veremos aquí, han requerido de la mayor creatividad e ingenio y dedicación por parte de los investigadores, quienes han puesto a prueba los enfoques y tecnología más modernos y variados.

Vacunas contra el SIDA

Importancia epidemiológica del Síndrome de la Inmunodeficiencia Adquirida

El SIDA es una enfermedad extendida en todo el mundo y sus tasas de incidencia y mortalidad van en aumento. Durante 1997 se infectaron en el mundo 5.8 millones de personas con el virus del SIDA (VIH). Se estima que a principios de 1998 había en el mundo 30.6 millones de personas VIH-positivas. Más de 40% de estas personas eran mujeres y la mitad de ellas tenía una edad de 15 a 24 años.[2]

Características generales del VIH

El VIH es un retrovirus. Su genoma esta constituido por RNA, el cual se replica por medio de un DNA intermediario de doble cadena, dentro de la célula blanco. El virus está envuelto por una cubierta por la membrana plasmática de la célula blanco que contiene glicoproteínas de cubierta (Gp 160 y Gp41), codificadas por el propio virus y que le sirven como medios de reconocimiento y anclaje a receptores específicos, en primera instancia a los macrófagos circulantes y después a los linfocitos $CD4^+$ Gp 41 es responsable de la fusión de la membrana del virus con la de la célula blanco [2]. Dentro de la cubierta se encuentra el genoma con un tamaño de 9.2-kbp y la transcriptasa reversa, encargada de sintetizar la doble cadena de DNA, usando como templado el genoma original del virus, inmediatamente después de la infección. [3] Desde el punto de vista inmunológico, el producto génico más importante es el de *env*, que codifica para las proteínas de cubierta Gp 120 y Gp 41.

Ciclo biológico de VIH-1

El virión VIH-1 rodeado de la glicoproteína de cubierta Gp 120 se une a dos co-receptores de superficie de los macrófagos circulantes: CD4 y CCR5.[4] Las membranas celulares se fusionan, y el virus entra a la célula. Entonces se liberan el genoma y la transcriptasa reversa virales. La transcriptasa reversa sintetiza una copia de DNA de doble cadena, la cual entra al núcleo y se integra al DNA celular. En este estado el VIH se llama provirus. Después se sintetizan y ensamblan las proteínas de VIH dentro de las células infectadas y el virus adquiere una cubierta lipídica, la cual contiene numerosas copias de Gp120 y Gp 41 y abandona las célula mediante un proceso de gemación.[5]

Vacunas contra el VIH[6]

El diseño de una vacuna capaz de proteger contra el SIDA sigue siendo un reto tan grande como cuando el virus estaba recién descubierto. Algunas de las razones más sobresalientes son las siguientes:

(a) La respuesta inmune natural no destruye al VIH y se forman reservorios de éste, principalmente en el cerebro, en células de la microglia.

(b) Ha sido necesario evitar el uso de virus completos vivos, atenuados o muertos, porque ésos representan un peligro potencial para las personas.

La proteína de cubierta Gp 120 puede mutar con el tiempo y unirse tanto a los co-receptores CD4 y CCR5 como a los timocitos portadores de $CD4^+$ (T CD4) , y de un cofactor llamado CXCR4 como a los cofactores CD4 y CCR5 de los macrófagos, como ocurre en la fase inicial de la enfermedad. En la fase tardía del SIDA la mayor parte de los virus pueden cambiar su afinidad exclusivamente por los correceptores de los linfocitos T $CD4^+$ y convertirse en "T-trópicos". En este estado los virus destruyen a las células T infectadas, contribuyendo fuertemente al colapso del sistema inmune y al surgimiento de la fase clínica de la enfermedad[4]. Actualmente se conocen seis co-receptores más, además de CCR5 y CXCR4. En todos los casos el virus VIH se une a CD4 y a uno de los otros factores.[2]

Vacunas para estimular la respuesta humoral

Este tipo de vacunas ha probado su eficacia para combatir enfermedades como la poliomielitis, el sarampión y la influenza. En el caso del SIDA los diseños de vacunas más sobresalientes son los siguientes:

Proteínas de cubierta (Env). La mayor parte de este tipo de vacunas contienen proteínas de la cubierta viral o parte de éstas, porque siendo estas proteínas necesarias para la unión del virus con la célula blanco, al unírsele anticuerpos específicos neutralizan su función y el virus es incapaz de infectar. Estas vacunas, dirigidas contra Gp 160, o contra Gp 120 y Gp 41 (que se derivan de la primera) ya han sido probadas en voluntarios humanos y se ha observado que se generan anticuerpos capaces de neutralizar virus vivos, eliminando su habilidad para infectar macrófagos humanos *in vitro*. Sin embargo, estos anticuerpos sólo reconocen a las cepas de VIH que se usaron en las vacunas. Cuando se usaron cepas aisladas de pacientes estas vacunas fueron ineficaces. Entonces, las proteías de cubierta puras parecen no ser los mejores candidatos para la vacuna.

Virus completos

Los virus completos, muertos, podrían presentar al sistema inmune una forma más natural de las proteínas de la cubierta, y por lo tanto estimular la producción de anticuerpos más eficaces para neutralizar la capacidad de infectar del VIH. Sin embargo, este enfoque implica el peligro de inocular eventualmente virus activos o el material genético residual, que es potencialmente peligroso. Por lo tanto deberá tenerse un especial cuidado para inactivar a los virus; y por otro lado, un tratamiento demasiado estricto para inactivar a los virus podría hacer a la vacuna menos efectiva, porque podría desprender y eliminar a las proteínas de la cubierta. Este es un buen enfoque, aunque deberá resolverse el problema de la estabilidad de las proteínas de cubierta.

Pseudoviriones. Estas son estructuras artificiales parecidas a los virus. Son esferas lipídicas vacías que sólo portan a la Gp 160. Estas estructuras son muy seguras porque no tiene la capacidad de infectar ni de hacerse reproducir por las células blanco. Sin embargo no es fácil fabricar pseudoviriones estables, aunque hay muchas esperanzas para lograrlo en un corto plazo.

Vacunas para estimular la respuesta celular

Las células citotóxicas reconocen epitopos. Los cuales son fragmentos de los antígenos presentados a los linfocitos T $CD8^+$ en la superficie de las células infectadas. Estos epitopos, en el momento de ser presentados, están ensamblados en los antígenos HLA, clase I. En este caso, no sólo los antígenos de superficie, sino también las proteínas internas pueden ser procesadas y presentadas a los linfocitos citotóxicos. En el caso de la vacuna contra el SIDA se deben seleccionar células específicas que sinteticen y expongan uno o más de los epitopos provenientes de las proteínas cuya síntesis es dirigida por el virus. De esta forma se podría inducir al sistema inmune a montar una respuesta contra todas las células infectadas que presenten en su superficie dichos epitopos.

Vacunas con vectores vivos. Esta modalidad de vacunas aprovecha la capacidad de ciertos virus (no VIH) para invadir celulas. La estrategia consiste en insertar genes del virus del VIH en estos virus, que no son patógenos para el hombre, permitiendo que estos agentes liberen dentro de las células los genes que codifican para los antígenos inductores de la respuesta inmune

protectora. La célula entonces produce las proteínas virales, las reduce a epitopos y estos son presentados en su superficie para activar a los linfocitos T, los cuales se activarán y formarán células de memoria, los que estarán listos para combatir una infección por VIH. Uno de los virus más usados para esta estrategia es el virus de la viruela de los canarios, un virus relacionado con el virus de la viruela humana, el cual ha sido transformado con los genes del VIH que codifican para las proteínas de la cubierta y para proteínas internas, como Gag (la proteína del núcleo viral y la proteasa. Estas vacunas son seguras y despiertan una débil respuesta celular. La respuesta se ha mejorado haciendo producir a los virus vectores un mayor número de copias o una mayor variedad de proteínas del HIV dentro de las células infectadas por el virus vacunal.

Péptidos. Se ha intentado despertar la respuesta inmune celular inyectando péptidos, pero éstos pueden degradarse antes de llegar a su destino. La inducción de la respuesta inmune por este tipo de moléculas podría incrementarse usando mejores adyuvantes.

DNA desnudo. En primates y ratones la vacuna ha estimulado la respuesta de linfocitos T y en algunos experimentos ha protegido a los animales de la infección.

Estrategias combinadas. Las estrategias más efectivas son aquellas que estimulan tanto a la respuesta celular como a la humoral. Por ejemplo, un programa de vacunación de éstos podría iniciarse con un virus de la viruela de los canarios portando el gen que codifica para las proteínas de cubierta, para estimular la respuesta celular, y meses después el mismo paciente podría recibir la Gp120 pura, para estimular la respuesta humoral. Con la primera vacuna se prepara a las células para una infección y con la segunda se refuerza.

Ya se ha hecho las primeras pruebas en seres humanos con este enfoque, pero se usaron antígenos de virus preparados en el laboratorio y se obtuvo una respuesta débil.

VIH y VIS modificados mediante ingeniería gética

Muchos investigadores siguen pensando que el uso de virus del VIH vivo es la mejor forma de inducir una respuesta protectora. Para ello han estado deletando sistemáticamente varios de los genes que este virus usa para replicarse. Un protocolo con médicos voluntarios se está llevando a cabo para probar esta vacuna.

Por otro lado se ha observado que el virus de la inmunodeficiencia de los primates, mal llamados simios (VIS) atenuados ha resultado seguro y demostrado su capacidad para inducir una respuesta protectora notablemente efectiva en estos animales. Sin embargo, en algunos casos estas vacunas han inducido síntomas de inmunodeficiencia, aún en animales no retados con el virus silvestre.

Vacunas contra amibiasis

La amibiasis es causada por *Entamoeba histolytica*. Se estima que afecta a 10% de la población mundial,[7] pero es muy probable que estas cifras tengan que reconsiderarse en un futuro cercano debido a que *E. Dispar* (una especie no patógena) también parasita al intestino humano, y hasta hace poco tiempo no se le distinguía de la primera.[8] Se estima que la amibiasis es causa 110 mil muertes al año.[9]

Las dos observaciones más generales acerca de que *E. histolytica* es capaz de despertar una respuesta inmune son: (a) que la frecuencia de reinfecciones por *E. histolytica* en pacientes recuperados de absceso hepático amibiano (AHA) es muy baja[11] y b) que los animales de laboratorio vacunados contra *E. histolytica* desarrollan una respuesta protectora contra abscesos hepáticos amibianos (AHA) o lesiones intestinales cuando se les inocula experimentalmente con amibas virulentas o disentería amibiana, lo cual discutiremos más adelante. Sin embargo el diseño de una vacuna confiable contra la amibiasis plantea una serie de dificultades, que deberán superarse para lograr el objetivo deseado.

Deben además tomarse en cuenta dos aspectos muy importantes de la biología de *E. histolytica*: su ciclo biológico y la capacidad que estos parásitos tienen para evadir la respuesta inmune.[5]

Es muy importante considerar el ciclo biológico completo del agente causal, como está sucediendo con el desarrollo de la vacuna contra la malaria[11] porque de esta manera se incrementa la posibilidad de identificar un mayor número de antígenos capa-

ces de despertar un respuesta inmune protectora y el número de oportunidades para interrumpir dicho ciclo. Los agentes causales de la malaria son varias especies de *Plasmodium*, cuyos ciclos biológicos[12] son mucho más complejos que el de *E. histolytica*. El ciclo biológico de *E. histolytica* consta solo de dos fases, una trófica, invasora y causante de la destrucción de tejidos (el trofozoíto) y otra infectiva, el quiste. El trofozoíto ha sido intensamente estudiado, pero la Biología Celular y Molecular de los quistes es prácticamente desconocida, fundamentalmente por la falta de un medio de enquistamiento masivo en condiciones axénicas para *E. histolytica*. Sin embargo se han hecho algunos avances en este sentido. En 1980 desarrollamos un medio eficiente, confiable y que además permite que las amibas inicien espontáneamente el enquistamiento en condiciones axénicas, pero con una pared débil.[17] Actualmente hemos desarrollado un nuevo medio donde las amibas forman una pared muy resistente, que contiene quitina y cuyas características morfológicas son prácticamente iguales a las de los quistes naturales. Pero en tanto no se obtenga un pleno éxito en los esfuerzos para producir quistes de *E. histolytca in vitro* todos los esfuerzos para desarrollar vacunas contra *E. histolytica* tendrán que seguirse concentrando en los antígenos de los trofozoítos.

Los trofozoítos de *E. histolytica* tienen un notable habilidad para evadir la respuesta inmune, inmovilizando,[14] degradando anticuerpos anti-antígenos de superficie de las amibas[15] y ejerciendo un efecto inmunosupresor. Esto último en los primeros estadios de la infección.[9] Por lo tanto, las vacunas que se desarrollen tendrán que considerar todas estas características de las amibas para lograr una respuesta inmune eficiente.

Las vacunas contra la amibiasis están en pleno desarrollo y todavía lejos de estar en condiciones de usarse masivamente en beneficio de la humanidad.

Considerando que tanto la respuesta humoral como la celular del sistema inmune son importantes, es posible que la vacuna más efectiva contra la amibiasis tenga también que estimular a ambas ramas del sistema inmune.

Algunos de los trabajos que enseguida citamos presentan sólo los resultados iniciales, que refuerzan la idea de que es posible obtener una respuesta humoral, tanto en la luz intestinal, como en el torrente circulatorio y también una respuesta celular protectora. Algunos otros presentan ya los resultados alentadores con vacunas experimentales en animales. A continuación presentamos algunos ejemplos de todos estos enfoques.

Inducción de la respuesta humoral utilizando trofozoítos completos usados como inmunógeno

Trofozoítos completos

Jain et al. Obtuvieron un cierto grado de protección en cobayos preinoculados con un número relativamente bajo de amibas vía intracecal y luego retados con un inóculo 80 a 100 veces mayor, también por vía intracecal o intramuscular.[16] Otros autores observaron incrementos significativos en los títulos circulantes[17] y coproanticuerpos[16] anti-*E. histolytica* después de inocular animales de laboratorio con trofozoítos fijados con glutaldehído. La respuesta inmune humoral se incrementa notablemente cuando se utiliza la toxina del cólera como adyuvante[18]

Transferencia de pasiva de inmunidad mediada por células

Las células peritoneales de hámsteres inoculados vía intradérmica con amibas vivas son capaces de proteger contra la infección experimental a hámsteres no inmunizados.[19]

Protección con anticuerpos monoclonales

Un anticuerpo monoclonal dirigido contra un lipofosfoglicano de *E. histolytica* llamado EH5 fue capaz de proteger contra AHA a ratones inmunodeficientes.[20]

Antígenos recombinanates

Uso de antígenos pre diseñados

MBP/SRHEP-C1A2 (SRHEP-H) es un antígeno que contiene fragmentos de las siguientes 3 proteínas: SRHEP (*Serin rich Entamoeba histolytica protein*), MBP (*maltose binding protein*) y la

subunidad de la toxina del cólera CTA_2, conocida por su capacidad de incrementar la respuesta inmune hacia otros inmunógenos. Esta vacuna molecular indujo un incremento en la producción de IgA en mucosas y de IgG en suero cuando se administró por vía oral a ratones.

Vacunas anti *E. histolytica* construídas en vehículos moleculares vivos.

Antígenos recombinantes expresados en *Salmonella typhi* atenuada.

Se transformó con un plásmido portador de una secuencia de DNA que codifica para un segmento de GalNac a *la cepa SL5928* de *Salmonella Dublín*. GalNac es una lectina localizada en la superficie de los trofozoítos de *E. histolytica* que se inhibe en presencia de galactosa/N-acetylglucosamina. Esta lectina juega un papel fundamental en la lisis por contacto que producen las amibas sobre sus células blanco. Cuando se inoculó por vía oral a un grupo de gerbos con estas bacterias se consiguió una disminución hasta de 90% en el tamaño de los abscesos hepáticos en comparación con los testigos no inmunizados.[22]

Zhang T y Stanley Jr. inmunizaron ratones con salmonelas vacunales typhi (Δcya, Δcrp, Δasd), las cuales tenían inactivos los genes que codifican para la adenil ciclasa (cya) y el receptor del AMP cíclico (crp). Esta cepa fue modificada mediante ingeniería genética para que sintetice SREHP/MBP. Los anticuerpos IGg anti-SREHP se incrementaron 10 veces en suero con respecto a los testigos no inmunizados. [23]

Algunas contribuciones de nuestro grupo

Nosotros hemos dedicado nuestro principal esfuerzo a la identificación y purificación de factores de virulencia de *E. histolytica* y al enquistamiento de este protozoario en condiciones axénicas. Ya antes comentamos la importancia de un método para enquistar masivamente *E. histolytica in vitro*. Enseguida comentaremos algunos de los avances que hemos tenido para identificar, purificar y clonar estos factores, los cuales podrían servir para investigar si estas proteínas amibianas completas o algunos de sus epitopos son capaces de inducir una respuesta inmune humoral o celular en animales de laboratorio.

Hemolisinas

Se ha sugerido que la actividad citolítica de *E. histolytica* juega un papel determinante en la habilidad que estos parásitos tienen para destruir a las células blanco. Nosotros encontramos que la actividad hemolítica en extractos libres de células esta relacionada con la virulencia de las cepas[24] Se han identificado varios factores citolíticos de *E. histolytica*, entre ellos una actividad dependiente del potencial de óxido-reducción[25] y fosfolipasas A.[26] También encontramos fosfolipasas A_1 y lisofosfolipasas en la misma fracción subcelular membranal de las amibas.[27] Las fosfolipasas A_1 son también citolíticas en algunas especies de protozoarios parásitos y las lisofosfolipasas inhiben la autodestrucción de las células destruyendo a los liso-derivados que las fosfolipasas A_1 y A_2 producen continuamente. Por estas características pensamos que estas tres enzimas son buenos candidatos para producir vacunas, induciendo la producción de anticuerpos contra estos factores de virulencia para inactivarlos y eventualmente despertar una respuesta celular contra las amibas portadoras de estos antígenos. Con esta idea en mente hemos purificado la principal fosfolipasa A_2 de *E. histolytica*, la cual es máxima a pH 8.4, y Ca^{2+} 1 mM[28] y clonamos una secuencia de cDNA que codifica para una citolisina dependiente de actividad de fosfolipasa A_2 (manuscrito en preparación). El siguiente paso será investigar si estas proteínas son capaces de inducir protección en animales de experimentación.

Por otro lado, investigaremos si los quistes que se forman espontáneamente en nuestro medio de cultivo (PEHPS) son capaces de inducir una respuesta inmune protectora contra amibiasis intestinal o absceso hepático amibiano en animales de experimentación.

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Archives of Medical Research

Entamoeba histolytica Is Unable to Use Free Cholesterol, Phospholipids, and Fatty Acids Under Axenic Cultivation Conditions

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Key Words: Entamoeba histolytica, Lipid auxotrophy, Lipid metabolism, Serum replacement.

Introduction

Diamond described, in 1978, a medium for the axenic cultivation of *Entamoeba histolytica*, called TYI-S-33 (1). Later, we developed another medium called PEHPS (2). The major constituents of these media are yeast extract, and liver and pancreas extract, respectively. Serum is an essential component of both. Preliminary data suggested that *E. histolytica* requires lipids to grow that are mainly supplied by serum. We recently described a protozoa axenic cultivation serum replacement (PACSR) (3) that contains Diamond's vitamin-Tween 80 mixture (1), amino acids, and a lipid-rich cholesterol preparation (LCR). This preparation contains lipoproteins, free cholesterol, phospholipids, and fatty acids (5). *E. histolytica* grew vigorously in PEHP or TYI basal media supplemented with PACSR for up to 3 years, but not in the presence of LCR, although these parasites survived for up to 72 h (3). This strongly suggests that *E. histolytica* requires an external source of lipids and/or lipoproteins, in addition to vitamins and amino acids. The objective of the present work was to determine whether *E. histolytica* is able to use free cholesterol, phospholipids, or fatty acids.

Materials and Methods

E. histolytica strain HK9 strain was used in this study.

Amebic cultures. Glass screwcap tubes (13 × 100 mm) containing 5 mL TYI-S-33 (1), PEHPS (2), PEHP, or TYI were supplemented with PACSR instead of bovine serum (4). Immediately afterward, 10^4 trophozoites/mL were inoculated and incubated for 72 h at 36°C. Subsequently, amebas were harvested and counted with an hemocytometer (2).

Experimental media. Tubes containing 5 mL PEHP were supplemented with Diamond's vitamin–Tween 80 mixture and Earle's amino acid solution (5) and 5 μL of the following lipids (dissolved in absolute ethanol): cholesterol (100 mg/mL); oleic (17 mg/mL), linoleic (58 mg/mL), linolenic (17 mg/mL), arachidonic (17 mg/mL), or docosahexanoic (17 mg/mL) acids; phosphatidylcholine (67 mg/mL); lysophosphatidylcholine (10 mg/mL), and sphingomyelin (26 mg/mL). Groups of three tubes were formed, and each was mixed with one of the lipid blends described in Table 1 or was added separately. All tubes were inoculated with 10^4 trophozoites/ml., incubated, and counted. All analyses were performed three times, in triplicate.

Results and Discussion

Trophozoites incubated in control media (containing 0.1% ethanol) grew well, reaching densities of 24.3 ± 3.7 in PEHPS and of 14.0 ± 2.68 in PEHP supplemented with PACSR. In the presence of any combination of free lipids, amebas died within 12 h of incubation. On the other hand, cultures maintained in media supplemented with 360 μg lipoproteins (5) reached densities quantitatively comparable to controls grown in PEHPS plus 0.1% ethanol (data not shown).

In conclusion, *E. histolytica* HK9 strain is unable to use free cholesterol, phospholipids, or fatty acids under axenic conditions. Nevertheless, as amebas were able to grow in the presence of lipoproteins under similar experimental conditions these parasitic protozoa possibly need an external source of

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resulting leaf cell suspension was filtered through gauzes of double thickness to remove residues. The cell suspension was washed three times with RPMI by centrifugation at 440 × *g* for 10 min. The pellet of leaf cells was suspended in a 20-fold volume of RPMI. Subsequently, the cells were disrupted by an ultrasonic processor (GE 50, Sonics and Materials, Inc.) for 30 min at 4°C. The extract was centrifuged at 15,000 × *g* for 20 min at 4°C and the supernatant fraction was sterilized by filtration (Millipore membrane filter; pore size, 0.22 mm). Ferredoxin content was assayed by the method of Buchanan and Arnon (5). Usually the sterilized fraction was added to YIGADHA-S at 0.3% (v/v) for cultivation of *E. dispar*. Further purification of ferredoxin was attempted by DEAE cellulose chromatography (5).

Results and Discussion

As previously mentioned, one strain (CYNO 16:TPC) began to grow in YIGADHA-S without any culture associates (protozoan, mammalian, plant, or other micro-organism cells) after a 3-month culture with 10% formalin-fixed *C. fasciculata* and a subsequent 3-month culture with 6-phosphogluconate, which is an intermediate metabolite of the pentose phosphate cycle and Entner-Doudroff pathway. However, four other strains (SAW1734R clone AR, CYNO 09:TPC, AS2IR, and AS16IR) needed a culture supplement, such as metabolically inactive sterilized *C. fasciculata*, for *in vitro* growth.

Based on these data, we attempted to examine the growth-promoting effect of plant ferredoxin, as iron–sulfur redox proteins are present in mitochondria, chloroplasts, hyrogenosomes, and bacteria, although with differences in the chemical structure of the iron–sulfur centers. We found that the supernatant fraction isolated from the extract of spiderwort leaf cells allowed successful cultivation of all five *E. dispar* strains at a similar growth rate (Figure1).

Determination of the ferredoxin content of the extract of spiderwort cells resulted in about 0.27 μmol/mL, which is equivalent to 3.2 mg/mL if the molecular weight (MW) of spiderwort ferredoxin is assumed to be 12,000, similar to spinach ferredoxin. Though measurement of ferredoxin content of the supernatant fraction of the spiderwort extract was not possible by the method employed, it could be purified from this fraction by DEAE cellulose chromatography, which was confirmed by spectrophotometric analysis. Thus, there is little doubt that the supernatant fraction contained ferredoxin. Preliminary studies on ferredoxin further purified by column chromatography demonstrated that it significantly promoted growth of *E. dispar* (SAW1734R clone AR, and AS2IR). To date (May, 2000), these five strains of *E. dispar* have been successfully subcultured for 9 months with the supernatant fraction of the spiderwort extract. Among these subcultures, one strain (CYNO 09:TPC) started to grow well in YIGADHA-S without supplementing the supernatant fraction, very similar to the axenic CYNO16:TPC strain.

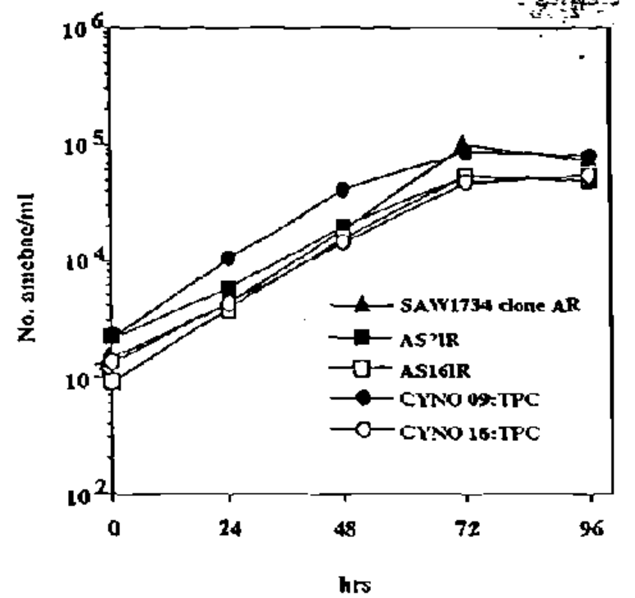

Figure 1. Growth kinetics of five strains of *E. dispar* supplemented with the supernatant fraction of leaf cell extract of spiderwort (*Commelina communis L.*) in YIGADHA-S.

Furthermore, the AS2IR and AS16IR strains are also being adapted to YIGADHA-S without adding the supernatant fraction. These results led us to envision that ferredoxin regulates one of the abilities of *E. dispar* for adaptation to the axenic culture conditions. Biochemical, molecular, and ultrastructural studies of these *E. dispar* strains are also being conducted.

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Table 1. Composition of media containing different combinations of free lipids[a]

Lipid blend	Lipids added to lipid test medium[b]								
	CHOL	PC	LPC	SM	LO	ARA	LNO	OL	DOCO
II	+	+	+	+	+	+	+	+	−
II	+	+	+	+	+	+	+	−	+
III	+	+	+	+	+	+	−	+	+
IV	+	+	+	+	+	−	+	+	+
V	+	+	+	+	−	+	+	+	+
VI	+	+	+	−	+	+	+	+	+
VII	+	+	−	+	+	+	+	+	+
VIII	+	−	+	+	+	+	+	+	+
IX	−	+	+	+	+	+	+	+	+
X	+	+	+	+	+	+	+	+	+

[a] Culture tubes containing 5 mL PEHP or TYI basal media supplemented with amino acids and vitamins were added with 5 µL of each lipid solution, as this table indicates. (+) = added; (−) = nonadded. The free lipids tested in these experiments are components of a lipid cholesterol-rich commercial preparation (LCR) and of a protozoa axenic cultivation serum replacement (PACSR). Their final concentrations were the same in experimental and in control media (PEHP/PACSR or TYI/PACSR); [b] The lipid test medium was PEHP basal medium supplemented with Diamond's vitamin–Tween 80 mixture and Earle's amino acid solution.

Abbreviations correspond to the following lipids: CHOL = cholesterol; PC = phosphatidylcholine; LPC = lysophosphatidylcholine; SM = sphingomyelin; LO = linoleic acid; ARA = arachidonic acid; LNO = linolenic acid; OL = oleic acid, and DOCO = docosahexanoic acid.

lipids structured as lipoproteins or integrated to cell membranes. These possibilities need to be further analyzed.

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Pathogenesis of *Entamoeba histolytica* Depends on the Concerted Action of Numerous Virulence Factors

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Key Words: Entamoeba histolytica, Virulence, Gal-lectin, Lipophosphoglycan, Antisense RNA, Transfection.

Introduction

Human infection with *Entamoeba histolytica* can take two paths: it can either develop an invasive form of the disease, or the infected individual may remain asymptomatic. In the last two decades, a number of virulence factors were identified and characterized in *E. histolytica*. The interaction between the amebic trophozoite and the mammalian target cells can be separated into two main stages: (a) the specific recognition and attachment of the parasite to the mammalian cell, which is mediated by a number of surface adhesins, the main adhesin being the well-studied Gal/GalNAc-specific lectin, and (b) a number of effector molecules, such as the amebapore and the cysteine proteinases, which can cause damage to host cells. Despite the remarkable progress of recent years, it is still not clear whether all the virulence factors that participate in causing the disease in humans have been identified. Furthermore, we still do not understand, in molecular terms, the variations that exist between *E. histolytica* strains of low and high virulence and the modulation of the expression of certain virulence factors in the same strain by external factors such as nutrients, bacteria, and/or other host factors.

Our recent results obtained from investigating (a) the avirulent *E. histolytica* strain Rahman, (b) the avirulent trophozoites of strain HK-9 monoxenically grown with *Escherichia coli* 055, and (c) the three avirulent transfectants of strain HM-1:IMSS in which the expression of a virulence factor was selectively inhibited by antisense RNA clearly indicate that pathogenesis in *E. histolytica* is the result of the concerted participation of numerous components.

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Materials and Methods

Avirulent E. histolytica *strain Rahman.* The three main virulence factors, i.e., the cysteine proteinases, the 170-kDa heavy subunit of the Gal/GalNAc-specific lectin, and the amebapore of the avirulent, axenically grown *E. histolytica* strain Rahman are all expressed at similar levels as in virulent strain HM-1:IMSS. On the other hand, a number of other components were found to be present in lower amounts in strain Rahman. One of the well-established characteristics of many pathogenic microbes is the presence of complex carbohydrate layers on their cell surfaces. Analysis of the ameba surface lipophosphoglycan (LPG) of strain Rahman have shown that it significantly differs from strain HM-1:IMSS in that it contains only 10% of the LPG content and is devoid of the long, branched (α1–6) glucose oligosaccharide chains (1,2). The lack of LPG in strain Rahman suggests that these complex macromolecules may play a role in amebic pathogenesis. The absence of branched oligosaccharide chains also suggests a mutational defect in the biosynthesis of LPG, and to identify putative inactive gene(s), we have compared by representation differential analysis (RDA) the identity and levels of RNA transcripts produced between strain Rahman and the well-established virulent strain HM-1:IMSS (3). Three genes were found to be underrepresented in strain Rahman. No clue is yet available for the downregulation of the first gene coding for an S-adenosylhomocysteine hydrolase. The second gene, which codes for the aldose 1-epimerase, has not yet been studied, and could perhaps be involved in the defect of the LPG molecules in trophozoites of the avirulent strain Rahman (see previous mention of this). The third underrepresented gene was the light subunit (35 kDa) of the Gal/GalNAc-specific lectin molecule that is linked, via an S-S bond, to the heavy (170-kDa) subunit, and has a GPI anchor to the membrane. Surprisingly, the levels of the heavy subunit (170 kDa) of the Gal/GalNAc-lectin and the adherence properties to mammalian cells of strain Rahman were similar to those found in virulent HM-1:IMSS. These findings suggested that the low expression of the light subunit could

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Identification of Seven Chemical Factors That Favor High-Quality *Entamoeba histolytica* Cyst-Like Structure Formation Under Axenic Conditions

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Key Words: Entamoeba histolytica, Encystation, Cysts, Simplex method.

Introduction

The biological cycle of *Entamoeba histolytica* consists of two stages: cyst (infective form), and trophozoite (invasive form). While the molecular and cell biology of trophozoites has been intensively studied, encystation is still poorly understood. This fact is mainly due to the absence of a culture medium that permits massive encystation under axenic conditions. We previously pointed out that *E. histolytica* spontaneously forms cyst-like structures (CLS) in PEHPS culture medium. The wall of most CLS did not resist the lytic effect of 0.4% sodium dodecyl sulfate (SDS) (1). Notwithstanding, when PEHPS was supplemented with an Mg^{2+} (1.22 mM), Mn^{2+} (14.44 mM), and Co^{2+}(19.44 mM) ion blend, the CLS wall resisted 1% SDS for 10 min, although these cells showed an irregular shape and a wrinkled surface (2). In this study, with the aid of the Simplex method (3), we analyzed the effect of 10 factors on high-quality CLS formation (HQ-CLS), under axenic conditions.

Materials and Methods

Amebas and cultivation methods. The *E. histolytica* HM-1: IMSS strain was used in all experiments. Twenty-five media were designed and tested with the aid of the Simplex method modified for multiple variables and multiple responses (3).

The study was performed in four steps. In the first, a matrix of 11 media containing all 10 chemical factors under study was tested, and the concentration of the factors producing the worst and best results was determined. During the study, the concentration of each factor in the previously mentioned media varied between a lowest and highest limit, chosen arbitrarily. The factors studied and their initial concentration limits were the following: bovine serum (0–1%); glucose (0–6%); sodium tetraborate (0–4 mM); $MgCl_2$ (1–4 mM); $MnCl_2$ (1–10 mM); $CoCl_2$ (1–10 mM); liver and pancreas extract (0–2.5%) (4); Diamond's vitamin mixture 107 (0–0.05%); phosphate (0–0.66 mM), and $CaCl_2$ (0–4 mM). To conform the second set of media, the 10 factors producing the worst results in step 1 were discarded, while the one that produced the best results was kept. By taking into account the concentration of each particular factor in media producing the worst and the best results, a second set of 10 new media were calculated. Results obtained with each media from the second set were compared with results obtained with the best media from the first set (3). By following this procedure, four additional media were discarded and substituted by four new media, which conformed the third set. The corresponding results were compared with the results of the former media, discarding only one medium. The substituting medium conformed the fourth set. The study was finished when the latter media produced worse results than those produced by any of the former.

All media were freshly prepared, using a basal medium (Casein peptone 2% in double-distilled water). This medium was distributed in 6-mL aliquots, in glass 16 × 125-mm screwcap tubes, and appropriate volumes of each of the previously described components were added, reaching a final volume of 12 mL. All tubes were gently stirred, immediately inoculated with 2×10^5 trophozoites, and incubated for 7 days at 37°C. All determinations were made in triplicate.

HQ-CLS evaluation. The biological quality of HQ-CLS produced in each of the previously described media was es-

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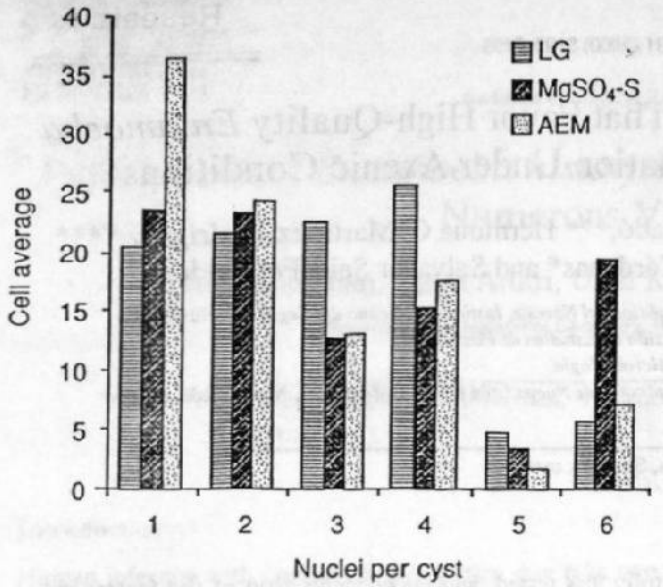

Figure 1. Quantification of the number of nuclei per cyst obtained with the different encystation media.

medium—69% in relation to inoculated trophozoites after 48-h incubation. With $MgSO_4$-serum, low cyst yields were attained (33%), and similar percentages were obtained with AEM (35%). However, with the latter significantly reduced trophozoite and total cell (trophozoites plus cysts) yields were observed. Low yields in AEM medium could reflect the destruction of trophozoites after transference to such a medium as a consequence of osmotic shock (70 mOsm). Thus, a decreased number of trophozoites remain viable and able to encyst. $MgSO_4$-serum medium appears to lack some crucial components, and the effect of temperature cannot be discarded.

The number of nuclei in cysts were counted to assess cyst maturity in different media. Higher percentages of tetranucleated cysts (25%) were attained with LG medium (Figure 1), immature cysts becoming gradually lower in these medium. On the other hand, uni- and binucleated cysts predominated in $MgSO_4$-serum medium, tetranucleated cysts being 15% of the entire population (Figure 1). Only 17% of cysts were tetranucleated in AEM (Figure 1), uninucleated cysts predominating in this medium. Cysts possessing five or more nuclei were observed in all media tested; they averaged 5% in LG medium, and were lower in the other two media. In some cysts, nuclei counting was impossible, especially in $MgSO_4$-serum medium (Figure 1). Differentiation of *E. invadens* in different media can afford us a better knowledge of events related with the amebic life cycle, including excystation.

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timated as a global quality index, which was defined as the geometric percentage average of the following five HQ-CLS characteristics: resistance to 0.1% sarcosyl lysis; rounded shape and a cell diameter of 8–20 μm; one or more well conserved nuclei; showing a smooth surface, and birefringence.

Results and Discussion

The Simplex is a very strong inductive method that allows procuring the best results by determining the relevant factors and their optimal concentrations in a particular biological system (3). Accordingly, in the present study we included several chemical factors that influence wall synthesis of diverse microorganisms and the main components of PEHPS to identify those that favored the HQ-CLS formation and their best concentration range in the culture medium. The factors that positively influenced HQ-CLS formation were the following: glucose (2–4%); boron (1–2 mM); Mg^{2+} (1–2 mM); Mn^{2+} (4–6 mM); Co^{2+}(6–10 mM); Ca^{2+} (2–4 mM), and bovine serum (0.5–1%). On the other hand, phosphates, vitamins, and liver and pancreas extract were irrelevant to HQ-CLS formation. Under these culture conditions, 55–60% of HQ-CLS were formed with respect to the inocula (2.1×10^6 trophozoites/mL). The microscopic appearance of HQ-CLS was remarkably similar to natural *E. histolytica* cysts. Figure 1 shows a spherical-shaped HQ-CLS having a birrefringent and smooth surface and a well-conserved nucleus, demonstrating a central centrosome and peripheral chromatine, and having a granular cytoplasm. HQ-CLS had a better appearance than CLS formed in PEHPS supplemented with Mg, Mn, and Co ions only (1) and, unlike CLS, formed spontaneously in aged cultures maintained in PEHPS (5) and resisted double-distilled water and 0.1% sarkosyl lysis. Nevertheless, the ultrastructure of these cells needs to be analyzed by electron microscopy and whether HQ-CLS are viable and capable to excyst needs to be investigated. In addition, the effect of physico-chemical factors such as pH, oxide-reduction potential, temperature, and osmolarity on *E. histolytica* encystation also needs to be analyzed.

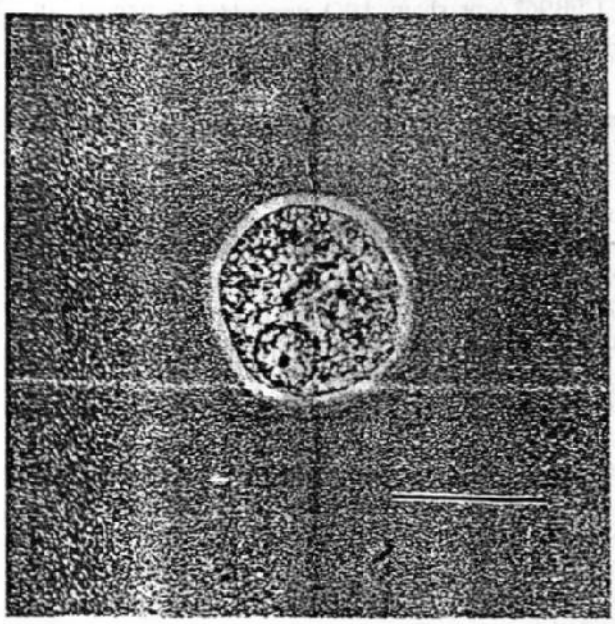

Figure 1. A high quality, cyst-like structure formed in a medium containing the seven factors favoring their formation under axenic conditions. These structures have a remarkable similarity to natural *Entamoeba histolytica* cysts, being spherical-shaped and having a smooth, birrefringent surface, a granular cytoplasm, and nuclei showing a peripheral chromatin and a central centrosome. Bar = 10 μm.

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ELSEVIER

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Incorporation of ^{14}C-Glucose into Cytoplasmic Glycogen from *Entamoeba invadens* IP-1 Strain

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Key Words: Entamoeba invadens, ^{14}C-Glucose, Glycogen, Cyst, Ameba.

Introduction

Glycogen represents the main intracellular source to obtain glucose in trophozoites of the *Entamoeba* genus (1). This carbohydrate is present in the trophozoite cytoplasm, and its concentration remains constant (2). However, in the cystic phase an important decrease in glycogen following cyst maturation has been observed (3). Previous reports concerning radioactive glucose transport were performed in ameba cultures to determine its participation in the different physiological conditions of this parasite (4,5).

The main goal of this study is to determine whether the radioactive glucose incorporated into trophozoites can be used as a precursor of the glycogen biosynthesis process. Standardization of the incorporation of radioactive glucose to intracellular glycogen granules of amebas could be used to identify whether these parasites degrade this polysaccharide when the cellular differentiation process has been initiated.

Materials and Methods

Ameba cultures. Axenic amebas of *Entamoeba invadens* strain IP-1 were used and kept in culture in 11 mL of Diamond's TYI-S-33 medium in 16 × 125-mm Pyrex tubes with screw caps. Each tube was inoculated with 10^4 amebas/mL, and was incubated at 25°C.

^{14}C-glucose incorporation by amebas. Trophozoites cultivated in TYI-S-33 medium were obtained after 7 days incubation at 25°C. The cells were transferred into Pyrex 16 × 125-mm tubes with screw caps that contained 10 mL of culture medium without glucose (this reagent was eliminated from the TYI-S-33 medium formulation). Each tube was inoculated with 10×10^3 trophozoites/mL and incubated at 25°C for 72 h. Tubes were centrifuged (1500 rpm, 15 min at 6°C), the supernatant was eliminated, and the pellet was transferred into tubes (Pyrex 10 × 75 mm with screw caps) that contained 3 mL of medium without glucose. A glucose mixture (140 mM nonradioactive glucose and 25 µCi D-U^{14}C-glucose, CFB96 Amersham, UK) was added in 250-µL aliquots to each tube and incubated at 25°C during 24 h.

Determination of ^{14}C-glucose incorporated into intracellular glycogen. After the incorporation of radioactive glucose by axenic trophozoites, the following procedures were carried out to determine whether they were able to integrate this radioactive substance into intracellular glycogen. Trophozoites were washed twice by centrifugation (1500 rpm for 10 min at 4°C) and resuspended in 2 mL PBS to determine the number of amebas. Two milliliters of 1% SDS was added to each tube to induce complete disruption of cells. The tubes were heated in a water bath for 15 min at 96°C, immediately cooled to 4°C, and 8 mL of distilled water was added. This procedure assured the absence of intact cells, which was confirmed under microscopic examination. The tubes were centrifuged (3000 rpm for 15 min at 4°C), the supernatant was eliminated, and the sediment suspended in 5 mL ethanol. Samples were boiled for 15 min and immediately cooled in water. They were then centrifuged at 3000 rpm for 15 min at 4°C, and the radioactivity present in the cell sediment was determined. The sediment was transferred to a glass flask containing 3 mL of biodegradable liquid scintillation (LSC-cocktail, Packard, Cat. 6013259). The amount of radioactivity was detected with 90% efficiency for carbon in a liquid scintillation analyzer (Packard, model 1600TR).

Results and Discussion

Trophozoites fasted of glucose for 72 h were capable of incorporating radioactive glucose in the following 24 h. Results obtained with about 32×10^3 amebas showed that after a 24 h period, radioactive glucose was incorporated into

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Mg^{2+}, Mn^{2+}, and Co^{2+} Stimulate *Entamoeba histolytica* to Produce Chitin-like Material

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ABSTRACT: The mechanism of *Entamoeba histolytica* cyst cell wall synthesis is not well understood. Previous research has shown that cyst-like structures formed in the presence of chitin synthase cofactors (Mg^{2+}, Mn^{2+}, and Co^{2+}) resist 1% sodium dodecyl sulfate lysis (RCLS), whereas those formed in the absence of cofactors (CLS) do not, and trophozoites are immediately destroyed. This suggests that *E. histolytica* is able to synthesize chitin during differentiation process under axenic conditions. To test this hypothesis, polysaccharide hydrolysates

from *E. histolytica* trophozoites. CLS, or RCLS were analyzed with high-performance liquid chromatography. The major components found in all 3 preparations were *N*-acetylglucosamine (NAG) and glucose (GLC), with RCLS possessing 129 and 180 times more NAG and 2.4 and 2.0 more GLC than trophozoites and CLS, respectively. After 36 hr of incubation with chitinase (16 U/ml) in a hypotonic medium (50 mOsm/kg), 68% of RCLS was lysed, and 100% lost affinity for calcofluor white M2R. The RCLS polysaccharides bound wheat germ agglutinin and appeared as long and thin or short and thick fibers. Accordingly, Mg^{2+}, Mn^{2+}, and Co^{2+} stimulated *E. histolytica* to synthesize a chitin-like material.

Entamoeba histolytica is the causative agent of amoebiasis. Its life cycle consists of trophozoite (invasive) and cyst (infective) phases. Trophozoites have been intensively studied, whereas the study of cysts is virtually nonexistent. This is mainly because of the absence of a medium that allows the mass encystation of amoebae under axenic conditions. A strategy to develop this medium would be to obtain an in vitro model where amoebae initiate their differentiation and then to analyze systematically each of the possible factors involved in the encystation mechanism. We previously reported that *E. histolytica* is able to form cystlike structures (CLS) in aged cultures maintained in PEHPS *(peptona de caseína, extracto de hígado y páncreas y suero)* medium (Said-Fernández et al., 1988). The microscopic appearance of CLS is remarkably similar to natural cysts, although the latter, but not CLS, resist hypotonic shock and detergent lysis (Said-Fernández et al., 1993). A possible cause of CLS weakness is a defect in the synthesis of chitin, assuming that chitin is present in the cell wall of *E. histolytica* cysts, as was suggested by Arroyo-Begovich et al. (1980). Chitin constitutes the matrix of the cell wall from fungi and *Entamoeba invadens*, conferring on them their characteristic resistance to adverse factors (Arroyo-Begovich et al., 1980; Ruíz-Herrera, 1982; Ruíz-Herrera et al., 1994).

Chitin is a polysaccharide formed exclusively by *N*-acetylglucosamine, with chitin synthase enzymes and their cofactors (Mg^{2+}, Mn^{2+}, and Co^{2+}) responsible for its synthesis (Ruíz-Herrera, 1982). We recently showed that, when trophozoites are incubated in PEHPS supplemented with these cofactors, amoebae form cystlike structures that are resistant to double-distilled water and 1% sodium dodecyl sulfate (RCLS), suggesting that these ions stimulate amoebae to produce chitin (Campos-Góngora et al., 2000). In the present study, the composition of polysaccharides produced by CLS and RCLS was determined and compared with the material obtained from trophozoites.

The *E. histolytica* HM-1:IMSS strain was used in this study. Amoebae were maintained under axenic conditions in PEHPS medium: 10 g casein peptone, 6 g glucose, 1 g L-cysteine, 0.2 g ascorbic acid, 0.6 g KH_2PO_4, 0.1 g K_2HPO_4, 250 ml bovine liver and swine and bovine pancreas extract, double distilled water as needed to 1 L and 9% (v/v) of bovine serum (Said-Fernández et al., 1988).

Three hundred culture tubes containing 11 ml PEHPS were inoculated with 1×10^5 trophozoites/ml and incubated at 36.5 C. One hundred tubes were harvested after 72 hr incubation to obtain trophozoites. Another 100 tubes were incubated for 10 days to obtain CLS (Said-Fernández et al., 1993), and the remaining 100 tubes were used to produce RCLS as follows. After 5 days of incubation, 10.5 ml of spent medium was substituted with an equal amount of fresh PEHPS medium. $MgCl_2$, $MnCl_2$, and $CoCl_2$ were added to produce final concentrations of 1.22 mM, 14.44 mM, and 19.44 mM, respectively; and then the cultures were incubated for 10 additional days. After being incubated at 37 C, the trophozoites, CLS, and RCLS were harvested by centrifugation at 700 *g* for 5 min and washed twice—trophozoites with 10 ml phosphate-buffered saline (PBS; pH 8.0) and CLS and RCLS with double-distilled water. Then, all preparations were freeze-dried in glass ampoules by applying a vacuum of 25 mtorr, at −50 C for 4 hr. Ampoules were sealed and stored at −20 C until used.

In all of the experiments related to carbohydrate analysis, a System Gold chromatograph (Beckman Instruments, Inc., San Ramon, California), equipped with a diode array detector and an ion-exclusion column (Aminex HPX-87H; Bio-Rad Laboratories, Hercules, California) was used.

A monosaccharide standards analysis was performed to test the capacity of the HPLC system to resolve several of the monosaccharides found in cell walls and to determine the retention times of each carbohydrate. Each compound (reagent grade, Sigma Chemical Co., St. Louis, Missouri) was dissolved in 5 mM H_2SO_4 (Merck, Darmstadt, Germany) at appropriate concentrations to obtain detectable peaks at an absorbance of 193 nm (about 0.02–2.5 absorbance units). The standards and their respective concentrations were as follows: 4.01 mg/ml fucose, 4.48 mg/ml mannose, 2.1 mg/ml glucosamine, 340 μg/ml *N*-acetylneuraminic acid, 300 μg/ml *N*-acetylmuramic acid, 70 μg/ml *N*-acetylglucosamine, and 4.7 mg/ml glucose. All the standard solutions were filtered through 0.45-μm Durapore filters (Millipore SA, de CV, México City, México), and 20 μl from each solution was injected into the Aminex column. A 5 mM H_2SO_4 isocratic elution, at a flow rate of 0.3 ml/min, was used. In addition, a solution containing all the monosaccharide standards was analyzed at the same final concentration of each individual solution. The retention time corresponding to each peak was matched with 1 of the standards injected separately to identify the corresponding compound.

The method described by Arroyo-Begovich et al. (1980), with minor modifications, was used to obtain amoebic polysaccharides. One hundred milligrams of freeze-dried trophozoites, CLS, or RCLS was resuspended in 5 ml double-distilled water and disrupted with 10 sonication cycles (75 W, for 30 sec, and 30 sec resting). The samples were centrifuged at 700 *g* for 15 min, and the pellet was washed 3 times with 10 ml double-distilled water. Each preparation was mixed with 1 ml of 0.5 N HCl and incubated at room temperature for 15 min, washed with 10 ml double-distilled water, and resuspended in 1 ml absolute ethanol : 1 N NaOH (2:1, v/v). Each of these preparations was heated in boiling water for 5 min and filtered through microfiber glass filters (GF/A, Whatman). The polysaccharides retained in the filters were eluted with 3 ml double-distilled water, freeze-dried as for the amoebic preparations, and stored until used.

The main monosaccharides contained in the amoebic polysaccharide hydrolysates were identified and quantified by using the external standard method (see Scott, 1995). The protocol consisted of 3 main steps: obtaining glucose (GLC) and *N*-acetylglucosamine (NAG) standard curves, determining the percentage of NAG recovered from chitin sample or NAG standard hydrolysates, and determining the contents of NAG and GLC in amoebic polysaccharide hydrolysates. Variable concentrations of NAG (3.62, 36.2, and 362 μg/ml) or GLC (48.0, 480,

charide hydrolysates could be underestimated by as much as 38%.

We have shown in this study that Mg^{2+}, Mn^{2+}, and Co^{2+} stimulate *E. histolytica* to produce a polysaccharide that has several of the chemical characteristics of chitin. In addition, this chitin-like material is mainly composed of fibers, similar to those observed in sagittal cuts from the *E. histolytica* cyst cell wall (Chávez et al., 1978). This strongly suggests that Mg, Mn, and Co ions stimulate *E. histolytica* to produce a chitin, which implies that *E. histolytica*, like *E. invadens* (Das and Gillin, 1991), has chitin synthase enzymes. Nevertheless, X-ray diffraction analysis of polysaccharides produced by RCLS, and the identification and cloning of the *E. histolytica* chitin synthase enzyme genes must be performed to confirm this possibility.

In addition to chitin, it is possible that another polysaccharide having GLC as a major component was produced by RCLS. It is highly unlikely that this molecule would be glycogen or cellulose, because glycogen is soluble in water (Budavari, 1996), and the procedure that we used to purify the amoebic polysaccharides included several washing steps with water. Furthermore, RCLS were not affected by cellulase. Thus, the GLC detected in our samples could belong to glucan(s) strongly bound to chitin, as in *Candida albicans* (Ruiz-Herrera et al., 1994).

The wall formed by RCLS is still abnormal (Campos-Góngora et al., 2000). This indicates that the Mg^{2+}, Mn^{2+}, and Co^{2+} concentrations that we used in this and previous studies are toxic to amoebae, and/or that other factors are involved in the *E. histolytica* cyst wall synthesis, such as possible inhibitory factors contained in PEHPS medium, the loss of genetic ability to encyst from the *E. histolytica* strain used in this and former studies, or the absence of some other factors needed to synthesize the cyst cell wall. All these possibilities are being investigated in our laboratory.

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TRICHOMONAS VAGINALIS: IDENTIFICATION OF A PHOSPHOLIPASE A–DEPENDENT HEMOLYTIC ACTIVITY IN A VESICULAR SUBCELLULAR FRACTION

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ABSTRACT: Trichomonad total extracts (TTE), or vesicular (P30) and soluble (S30) subcellular fractions from 3 pathogenic *Trichomonas vaginalis* strains (GT-3, GT-13, and GT-15), lysed both human and Sprague–Dawley rat erythrocytes in a time- and dose-dependent manner. The entire hemolytic activity of TTE was located in P30, showing 2 peaks of maximum activity, one at pH 6.0 and another at pH 8.0, in the presence of 1 mM Ca^{2+}. Hemolytic activity on rat erythrocytes was greater at pH 6.0 (6.71 ± 0.33 hemolytic units [HU]/mg/hr to 11.60 ± 0.24 HU/mg/hr) than at pH 8.0 (3.81 ± 0.30 HU/mg/hr to 5.75 ± 0.65 HU/mg/hr), and it was greater than that on human red blood cells at pH 6.0 (2.67 ± 0.19 HU/mg/hr to 4.08 ± 0.15 HU/mg/hr) or pH 8.0 (2.24 ± 0.09 HU/mg/hr to 2.81 ± 0.66 HU/mg/hr). The alkaline and acidic hemolytic activity diminished (60–93% at pH 6.0 and 78–93% at pH 8.0) by the effect of 80 μM Rosenthal's inhibitor, which also inhibited 27–45% and 29–54% trichomonad alkaline and acidic phospholipase A activities, respectively. Vesicles, vacuoles, and hydrogenosomes were rich in P30. *Trichomonas vaginalis* has a hemolytic PLA, which could be involved in its cytopathogenic mechanism.

Trichomoniasis affects 12% of men and 16% of women worldwide. It is caused by sexually transmitted *Trichomonas vaginalis* and can either run an asymptomatic course or be manifest as vaginitis, urethritis, or both. This parasitic disease has been suggested as a factor in increasing the probability of developing cervical neoplasia (Zhang and Begg, 1994) and favoring the progression of human immunodeficiency virus (Krysvac, 1992). It has also been associated with intrauterine growth retardation (Germain et al., 1994) and male infertility (Moskowitz and Mellinger, 1992).

Trichomonas vaginalis has the ability to destroy in vitro monolayers of epithelial cells isolated from human vaginal mucosae. Phagocytosis (Rendon-Maldonado et al., 1998), cytolysis (Gilbert et al., 2000), hemolysis (De Carli et al., 1996), and mammalian cell monolayer disruption (González-Robles et al., 1995; Gilbert et al., 2000) are important components of trichomonad cytopathogenicity, which is closely related to virulence of these (De Carli et al., 1994, 1996; Nagao et al., 2000) and other parasitic protozoa, i.e., *Entamoeba histolytica* (Ravdin, 1988), *Trypanosoma cruzi* (Wainszelbaum et al., 2001), *Acanthamoeba castellanii* (Ferrante, 1991), *Naegleria fowleri* (Barbour and Marciano-Cabral, 2001), and *Plasmodium falciparum* (Pouvelle et al., 1995).

As in the case of *E. histolytica* (Ravdin, 1988), cytolysis produced by trichomonad depends on parasite contact with target cells (Gilbert et al., 2000). Notwithstanding, some workers have pointed out that *T. vaginalis* and *T. foetus* also are capable of releasing soluble cytolysins and hemolysins (Burgess et al., 1990; Fiori et al., 1996).

Factors related to cytopathogenicity of *T. vaginalis* and other trichomonad species are surface glycoproteins, responsible for parasite adherence to target cells (Alderete et al., 1986), neuraminidase (Dias Filho et al., 1999) and proteinases that contribute to detaching tissues and cultured monolayers (Arroyo and Alderete, 1995; Alvarez-Sánchez et al., 2000), and pore-forming proteins (Fiori et al., 1996) that lyse erythrocytes and nucleated cells.

Phospholipases A (PLA) have not yet been identified as virulence factors in pathogenic trichomonad species, even after these enzymes have been identified as significant virulence factors in other parasitic protozoa such as *E. histolytica* (Long-Krug et al., 1985; Vargas-Villarreal et al., 1995; González-Garza et al., 2000), *T. cruzi* (Wainszelbaum et al., 2001), and *N. fowleri* (Barbour and Marciano-Cabral, 2001), and as the main cytolysins of insect, arachnid, and reptile venoms (Dennis, 1983).

Most *T. vaginalis* cytopathogenic factors have been detected by using in vitro models in which living trichomonads or spent culture medium were coincubated with target cells. Nevertheless, the effects of incubation time, dose, pH, and ion concentration have often been disregarded, knowing that some of these chemical and physicochemical factors are usually essential in identifying and characterizing enzymatic activities related to cytopathogenicity, i.e., proteinases (Ravdin, 1988; Bozner and Demes, 1991) and phospholipases (Dennis, 1983). Moreover, pore-forming proteins (Fiori et al., 1996) and lectins are also dependent on some of these factors (Goldstein and Gillin, 1986). This could explain why some (Burgess et al., 1990; Pindak et al., 1993; Fiori et al., 1996) but not other (Dailey et al., 1990; De Carli et al., 1994) researchers have been able to detect the effects of soluble hemolysins and why phospholipases have not yet been identified as trichomonad hemolytic or cytolytic factors. In addition, systematic analyses of identifying subcellular fractions that contain hemolytic or cytolytic trichomonads have not been performed, knowing whether or not cytolysis is associated with membranal structures such as *T. histolytica* PLA (González-Garza et al., 2000) or *T. vaginalis* CP65 cysteine proteinase (Alvarez-Sánchez et al., 2000). This knowledge could contribute to understanding the manner in which these cytotoxins enter into contact with target cells and to facilitating the strategy for their isolation and further characterization.

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Accordingly, in the present study we investigated whether *T. vaginalis* cell–free total extracts from 3 pathogenic strains (GT-3, GT-13, and GT-15) have hemolytic activity and also determined the subcellular distribution of this activity and identified several physicochemical factors that allow its maximum expression. In addition, we investigated if this activity depends on PLA activity and analyzed the ultramicroscopic structure of the trichomonad subcellular fraction that contained the complete hemolytic activity of these parasites.

MATERIALS AND METHODS

Parasites

Trichomonas vaginalis (GT-3, GT-13, and GT-15 strains) were used in all the experiments, except in those concerning an analysis of the ultrastructure of P30, pH, and Ca^{2+} dependence of P30's hemolytic activity, where only GT-13 was used. All 3 trichomonad strains were kindly donated to us by Dr. Fernando Anaya-Velázquez, from the Instituto de Biología Experimental, Facultad de Química, Universidad de Guanajuato, México. These strains were isolated in Guanjuato State, México, from vaginal secretions of patients having symptomatic trichomoniasis (Padilla-Vaca and Anaya-Velázquez, 1997). Immediately on arrival at our laboratory, the trichomonads were placed in PEHPS medium (Castro-Garza et al., 1996; Mata Cárdenas et al., 1998) and subcultivated twice a week, for 2 mo, throughout the experiments or cryopreserved until use to avoid changes in their original biological properties, as described below.

Cultivation methods

Preparation of PEHPS medium: The medium components were combined (175 ml bovine liver and bovine and swine pancreas extract [Said-Fernández et al., 1988], 5.0 g casein peptone, 3.0 g glucose, 0.5 g L-cysteine, 0.1 g ascorbic acid, 0.2 g NaCl, 0.3 g KH_2PO_4, and 0.5 g K_2HPO_4), resuspended with double-distilled water, the pH adjusted to 7.0, and the volume brought up to 500 ml. This medium was distributed in 10-ml aliquots in 16- × 125-mm screw capped borosilicate culture tubes and sterilized at 121 C for 15 min; 1 ml sterile bovine serum was added to each tube. The medium was used immediately (Said-Fernández et al., 1988).

Trichomonad subcultivation: Three tubes containing 10 ml PEHPS plus 10% (v/v) bovine serum and Diamond's vitamin–Tween 80 mixture (Diamond et al., 1978) were inoculated with 1×10^3 trichomonads/ml and incubated at 36.5 C for 96 hr. Each tube was observed with an inverted microscope, and the one containing protozoa with the highest density and mobility was cooled in ice water for 10 min. The number of trichomonads per milliliter was then determined with a hemacytometer. The trichomonads were inoculated, as above, into 3 tubes containing fresh culture medium. This procedure was repeated systematically every 96 hr to maintain the reference strain (Castro-Garza et al., 1996; Mata-Cárdenas et al., 1998).

Mass cultures: To perform each experiment, 3 spinner flasks (Bellco Glass Inc., Vineland, New Jersey) containing 600 ml PEHPS, supplemented with 10% (v/v) bovine serum and Diamond's vitamin–Tween 80 mixture (Diamond et al., 1978), were inoculated with 1×10^5 trichomonads/ml and incubated steadily at 36.5 C for 96 hr. The cultures were chilled in ice water for 10 min and centrifuged at 1,000 *g* for 15 min at 4 C. Protozoa were washed twice with 10 volumes of phosphate-buffered saline (PBS; pH 7.0) and processed immediately after obtaining the trichomonad total extracts (TTE) or the subcellular fractions.

Cryopreservation

Trichomonads (2×10^5, growing logarithmically) were collected, centrifuged (1,000 *g* for 15 min at 4 C) and resuspended with 1 ml of 10% dimethyl sulfoxide (DMSO) in fresh LYFS-33 medium (Diamond et al., 1978). Preparations were put into 2 ml cryo-vials (Sigma Chemical Company, St. Louis, Missouri), kept at 36.5 C for 15 min, and frozen at −20 C for 2 hr and then at −70 C for 2 hr more before being placed in liquid nitrogen. Two cryo-vials containing frozen trichomonads were thawed in a water bath at 36.5 C immediately before use, and trophozoites from each vial were inoculated into tubes containing 10 ml PEHPS medium (Said-Fernández et al., 1988). Twenty hours later the trichomonads were reinoculated in fresh PEHPS medium. The successive reinoculations were performed every 96 hr.

Obtaining TTE and subcellular fractions

The hemolytic activities of TTE, S30, and P30 were assayed at various pH values (5.0–9.5) or solely at pH 6.0 and pH 8.0, as described. Different batches of TTE, S30, or P30, having one of the aforementioned pH values, were prepared as follows. A pellet of freshly obtained trichomonads was washed, resuspended, fractionated, and diluted with Hank's balanced salt solution (BSS; 0.7 mM $CaCl_2$, 5.5 mM glucose, 120 mM NaCl, 5.3 mM KCl, 1.7 mM $MgSO_4$, 1 mM Trizma base). The pH was brought to the desired value by addition of appropriate concentrations of sodium acetate–acetic acid (pH 5.5, 6.0, and 6.5) or Trizma base (pH 7.0–9.5). A freshly obtained pellet of trichomonads was washed once with 10 volumes PBS and centrifuged at 1,000 *g* for 15 min at 4 C. The pellet was resuspended with 1 volume BSS and the trichomonads disrupted with a motor-driven Elvehjem Potter Teflon/glass homogenizer (Yarlett et al., 1993). Three milliliters of TTE were saved, divided into 0.5-ml aliquots, and stored in liquid nitrogen until required. The remaining TTE was centrifuged at 30,000 *g* for 15 min at 4 C. The resultant supernatant (S30) was stored until use. The pellet (P30) was resuspended with 1 volume BSS, distributed in 200-µl aliquots, and stored at −70 C. Immediately before the start of each experiment, a sufficient number of TTE, P30, and S30 aliquots were thawed at room temperature and diluted with BSS to adjust the protein concentration to 1–16 mg/ml, according to each experiment design (see below).

Hemolysis assays

Hemolysis assays were performed by adapting experimental conditions to a method described previously to analyze the hemolytic activity of *E. histolytica* subcellular fractions (Said-Fernández and López-Revilla, 1982). Erythrocyte suspensions with predetermined pH values and Ca^{2+} concentration were used according to the design of each experiment. Twenty-five microliters of human (group O Rh+, from a healthy donor) or Sprague–Dawley rat erythrocyte suspensions, preadjusted at 300 mOsm/kg and to an optical density of 0.8 at 415 nm, were placed into 1.5-ml-capacity polypropylene centrifuge tubes (Sigma) and mixed with 25 µl of TTE, S30, or P30. In addition, 3 tubes were treated with 25 µl double-distilled water or 25 µl BSS instead of the trichomonad extracts, which were used as 100% and 0% hemolysis controls, respectively. The hemolysis mixtures were incubated at 36.5 C for various periods of time (see below). Subsequently, 1 ml PBS was added and the cultures centrifuged at 600 *g* for 5 min at 4 C. The absorbance at 415 nm (A_{415}) was measured with a spectrophotometer (PMQ III, model MM3, Zeiss, Oberkochen, Germany). The percentage of hemolysis in each tube was determined by applying the following equation: % He = (ExHR − SHR)/(MHR − SHR) × 100, where % He is the hemolysis percentage, ExHR the experimental hemoglobin release, SHR the spontaneous hemoglobin release (in mixtures with added BSS instead of trichomonad extracts), and MHR the maximum hemoglobin release (in mixtures treated with double-distilled water instead of trichomonad preparations). One hemolytic unit (HU) was defined as the amount of protein from TTE, or any of the subcellular fractions, required to produce 50% hemolysis (HD_{50}) in the aforementioned assay mixtures. These data were interpolated in the respective dose–response curves. The specific hemolytic activity of the trichomonad preparation was expressed as the amount of HU per milligram of protein for 1 hr incubation at 36.5 C (HU/mg/hr).

Effects of extract dose, incubation times, Ca^{2+} concentration, pH, and Rosenthal's inhibitor on the hemolytic activity of trichomonad extracts

All these experimentations were performed following the hemolysis method previously described, with the following exceptions. The effect of pH was determined with hemolysis mixtures containing rat erythrocytes, a P30, at a dose equivalent to 200 µg of protein. The hemolysis mixtures containing 1 mM Ca^{2+} were incubated for 2 hr at 36.5 C, with a series of pH values ranging from 5.5 to 9.5. The Ca^{2+} effect was analyzed in hemolysis mixtures containing rat erythrocytes, 200 µg of P30 proteins, and 0–3.5 mM $CaCl_2$. The assays were carried out at both

pH 6.0 and pH 8.0. The incubation times were 1 and 2 hr, respectively. To determine the effects of incubation time and dose–response curves, hemolysis mixtures containing rat or human erythrocytes and 1 mM Ca^{2+} were used. The temporal relationship of hemolytic activity was determined by using 25 µl of P30 (having 200 µg proteins), TTE, or S30 (containing 400 µg proteins). These assays were carried out at both pH 8.0 and pH 6.0. Dose–response curves were used to calculate the specific hemolytic of trichomonad preparations. These were determined by testing a series of TTE, S30, or P30 doses (having 0–400 µg protein). Hemolysis mixtures were incubated for enough time to obtain at least 80% hemolysis or for 5 hr maximum, i.e., TTE and S30 hemolysis mixtures were incubated for 5 hr, P30 with rat erythrocytes for 2 hr at pH 8.0 and 1 hr at pH 6.0 and with human erythrocytes for 5 hr at pH 8.0 and 2.5 hr at pH 6.0.

To analyze the effect of a PLA inhibitor on hemolytic activity, P30 samples containing 200 µg protein were mixed with various doses (0–80 µM) of Rosenthal's inhibitor (dimethyl-DL-2,3-distearoyloxypropyl-2'-hydroxyethylammonium; Sigma). The mixtures were incubated for 30 min at 36.5 C, and immediately afterward 25 µl of a 2% (v/v) rat erythrocyte suspension (pH 6.0 or 8.0) was added. Each hemolysis preparation was mixed thoroughly with a vortex and incubated again for 1 hr (pH 6.0 preparations) or 2 hr (pH 8.0 preparations) at 36.5 C.

Phospholipase A assays

These assays were performed as described previously (Vargas-Villarreal et al., 1995), with minor modifications. In 1.5-ml cone-bottom borosilicate vials (Bellco Glass) were mixed 1.0 ml of 100 mM sodium acetate–acetic acid (pH 6.0) or 100 mM of Trizma base (pH 8.0), 2 mM Ca^{2+}, 0.2% Triton X-100, 0.27 mM phosphatidylcholine, and 4 µCi of 1,2-dipalmitoyl-sn-glycero-3-phosphoryl-[choline-methyl-^{3}H]-choline per milliliter ([^{3}H-choline]-PC; 60 Ci/mmol; New England Nuclear, Boston, Massachusetts). The mixtures were sonicated with an Ultratip Labsonic System (Lab-Line Instrument Inc., Melrose Park, Illinois), which was operated at 40 W for 60 sec. The resultant emulsion was divided into 0.5-ml aliquots in vials and stored at −70 C until required.

The assays were performed in 15- × 15-mm borosilicate test tubes, in which 10 µl of 1 of the above-described substrate mixtures was added, and mixed with 10 µl of the P30 of GT-3, GT-13, or GT-15 suspension containing 200 µg of total protein. After 1 hr (pH 6.0) or 2 hr (pH 8.0) of incubation at 36.5 C in a water bath, phospholipid hydrolysis was stopped by addition of 25 µl of 1 mg egg yolk lysophosphatidylcholine (LPC)/ml and 0.75 mg egg yolk phosphatidylcholine (PC)/ml in 5% trichloroacetic acid in *n*-butanol to bring the mixture to a final volume of 45 µl.

The radioactivity in PC and LPC from the assay was determined and separated by thin-layer chromatography as follows. On 20- × 20-cm silica-gel plates (0.25-mm thickness, 60-mesh; Merck, Darmstadt, Germany) was poured, drop by drop, 45 µl of the above-described mixture, at the origin of the chromatograms. The plates were placed into a thin-layer chromotograph (TLC)–developing tank with a solvent system of chloroform–methanol–acetic acid–water (140:40:16:8, v/v/v/v). Lipid spots were developed by exposing the TLC plates to iodine vapor (Skipsky and Barclay, 1969).

Identification of PC and LPC was performed by comparing the appearance and relative migration coefficients (Rf: PC, 0.43 and LPC, 0.185) of spots from experimental samples with those of the corresponding standards (Sigma). The lipid spots from each lane (developed with iodine vapor) were scraped, the released material put into vials containing 5 ml of scintillation liquid (biodegradable counting scintillant, Amersham Corporation Arlington Heights, Illinois), and its radioactivity determined by a 3,255 Tri-Carb liquid scintillation spectrometer (Packard Instrument Company, Incorporated, Downers Grove, Illinois), equipped with an external standard source (^{226}Ra[γ] assembly, and preadjusted to analyze the relatively unquenched samples. Specific PLA activity (PLAU/mg/hr) was calculated by defining arbitrarily 1 unit of PLA (PLAU) as the hydrolysis of 1 nmol of [^{3}H-choline]-PC/mg protein/hr.

Effect of Rosenthal's inhibitor on trichomonads PLA activity

Assay mixtures containing 200 µg P30 total protein of GT-3, GT-13, or GT-15 and 80 µM of Rosenthal's inhibitor were incubated for 30 min at 36.5 C. Immediately afterward, 4 µCi [^{3}H-choline]-PC/ml dissolved in 0.020 ml of 100 mM sodium acetate–acetic acid (pH 6.0) or 100 mM of Trizma base buffer (pH 8.0), 1 mM Ca^{2+}, and 0.1% Triton X-100 were added. Each preparation was mixed thoroughly with a vortex and incubated again for 1 hr (pH 6.0 preparations) or 2 hr (pH 8.0 preparations) at 36.5 C. The hydrolysis products ([^{3}H]-LPC) were separated by thin-layer chromatography and quantified as described above.

Protein determination

Protein concentration was determined according to Lowry et al. (1951).

Electron microscopy

A P30 sample (200 µl) was fixed with 1 volume of 5% (v/v) glutaraldehyde for 1 hr at 36.5 C and centrifuged at 310 *g* for 5 min at 4 C. The pellet was washed (twice with 0.1 M cacodilate buffer (pH 7.4) and resuspended in 1% (w/v) osmium tetraoxide. The sample was dehydrated with a series of ethanol concentrations (60–100%) and embedded with Epon Epoxic Resin (Pelico, Redding, California). The electron micrographs were obtained with a Carl Zeiss EM-109 electron microscope (Zeiss).

Statistical analysis

All results are the average and standard error (SE) of 3 experiments, each performed in triplicate. Where indicated, the association between dose–response hemolysis and PLA curves was calculated by using the 2-tailed lineal correlation simple method, with the aid of the Statistical Package for Social Science (SPSS for Windows, Standard Version 10.0), with 95% confidence. In addition, the means of HD$_{50}$ determined (in triplicate) in GT-3, GT-13, and GT-15 were compared by using the 1-way analysis of variance test.

RESULTS

Dependence of P30's hemolytic activity on pH, and Ca^{2+} concentration

Hemolytic activity of P30 presented 2 peaks of maximum activity as a function of pH, one at pH 6.0 and the other at pH 8.0 (Fig. 1). The difference between hemolysis obtained at pH 6.0 and pH 8.0 was highly significant with respect to that determined for all other pH values ($P < 0.005$). Hemolysis produced at pH 6.0 and pH 8.0 rose as a function of Ca^{2+} concentration from 0.5 to 1 mM. Higher Ca^{2+} concentrations inhibited both alkaline and acidic hemolytic activities (Fig. 2). The differences between hemolysis produced by P30 containing 1 mM Ca^{2+}, at both pH 6.0 and pH 8.0, were highly significant ($P < 0.005$) with respect to that produced by P30 preparations containing any other Ca^{2+} concentration, except for those which were assayed at pH 8.0 and contained 1.5 mM Ca^{2+} (P [illegible]).

Time relationship of hemolytic activity in TTE and subcellular fractions

Trichomonad total extracts from GT-3, GT-13, GT-15 were capable of lysing both human and rat erythrocytes at pH 8.0 or at pH 6.0. In all cases the hemolysis observed up to the first hour of incubation [illegible] and the 5th hour, the range of hemolysis produced in human erythrocytes at pH 6.0 or 8.0, or against rat erythrocytes at pH 8.0 did not surpass 31.5% with respect to 100% lysis, whereas the hemolytic effect of TTE at pH 6.0 against human erythrocytes increased with time, reaching 78–99% lysis, 2.5–4.0 times higher than under the aforementioned conditions. The hemolysis produced against rat erythrocytes at pH 6.0 and at pH 8.0 by using [illegible] P30 increased with respect to incubation time [illegible]

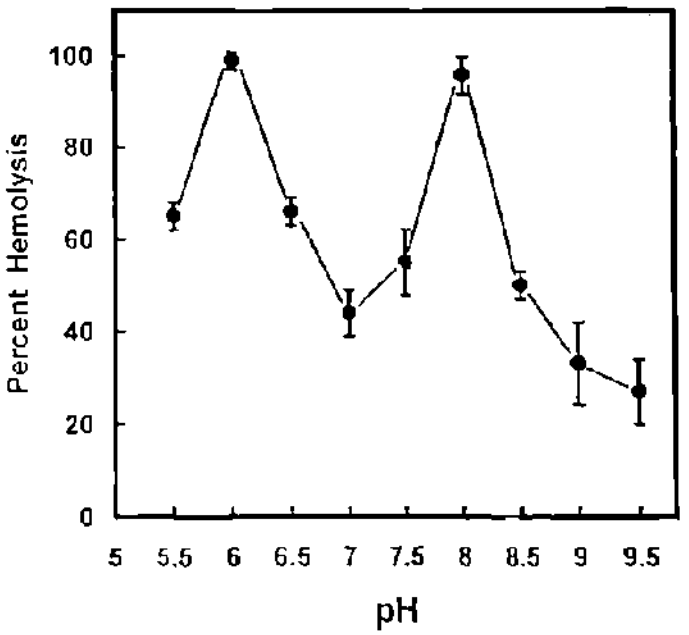

FIGURE 1. Effect of pH on P30 hemolytic activity. The hemolytic activity of P30 doses equivalent to 200 μg proteins at several pH values was analyzed using rat erythrocytes. The samples were incubated for 2 hr at 36.5 C. Symbols correspond to the averages ± SE of 3 experiments in triplicate. The difference between percent hemolysis observed at pH 6.0 and 8.0 was highly significant with respect to that determined at all other pH values ($P < 0.005$).

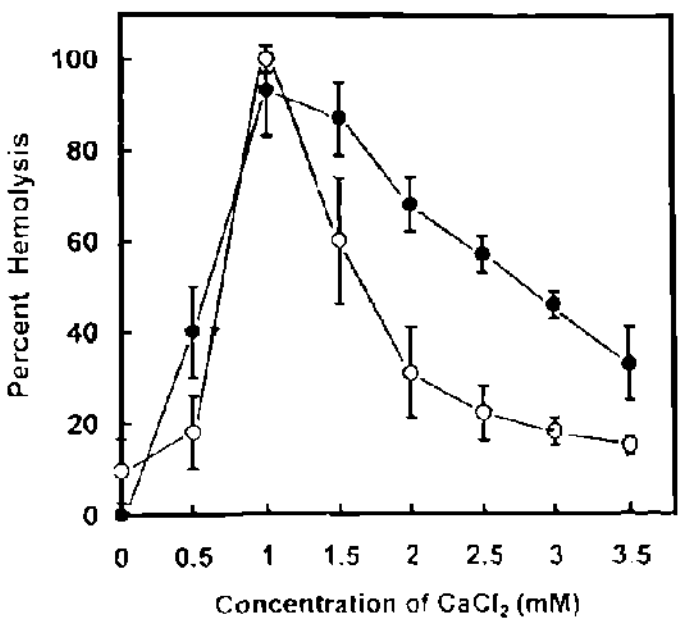

FIGURE 2. Calcium dependence of P30 hemolytic activity. Assay mixtures contained 200 μg of P30 proteins and various $CaCl_2$ concentrations. The assays were carried out at pH 6.0 (○) and pH 8.0 (●). Assay mixtures with pH 6.0 were incubated for 1 hr and those with pH 8.0 for 2 hr. All experiments were incubated at 36.5 C. Symbols correspond to the averages ± SE of 3 experiments in triplicate. Differences between hemolysis produced by P30 containing 1 mM Ca^{++} at both pH 6.0 and pH 8.0, were highly significant ($P < 0.005$) with respect to that produced by P30 preparations containing any other Ca^{++} concentration, except for those that were assayed at pH 8.0 and contained 1.5 mM Ca^{++} ($P = 0.3$).

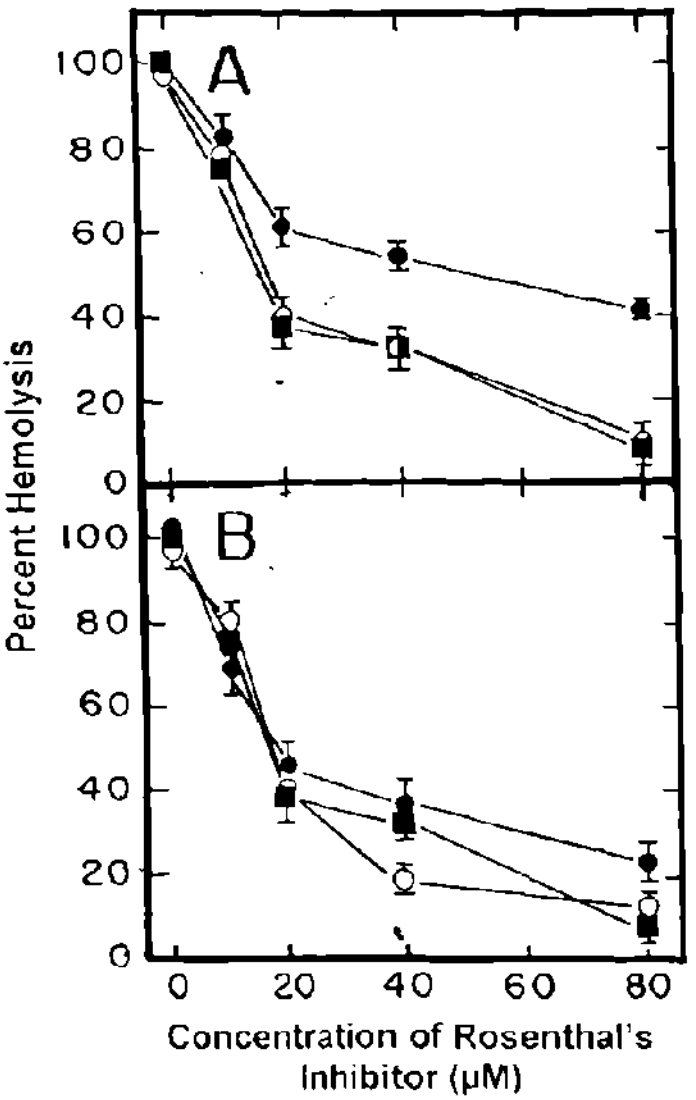

FIGURE 3. Effect of Rosenthal's inhibitor on the hemolytic activity of P30. Ten microliters of P30 from strains GT-3 (○), GT-13 (●), or GT-15 (■) were mixed with various doses of Rosenthal's inhibitor and incubated at 36.5 C for 30 min. Immediately afterward, 25 μl of 2% (v/v) rat erythrocytes were added and reincubated at 36.5 C, those mixtures with pH 6.0 for 1 hr (frame A) and those with pH 8.0 for 2 hr (frame B). Each point corresponds to the averages ± SE of 3 determinations in triplicate. The P30 hemolytic activity of GT-13 was more susceptible to the effect of Rosenthal's inhibitor at pH 8.0 than at pH 6.0 at all concentrations ($P < 0.05$).

98%. Maximum rat red blood cell hemolysis took 1 hr at pH 6.0 and 2 hr at pH 8.0. Whereas that of human erythrocytes lasted for 2.5 hr at pH 6.0, and 5 hr at pH 8.0 (Fig. 3). S30 had no detectable hemolytic activity.

Dose-dependence of TTE and P30 specific hemolytic activity

Hemolysis produced by TTE from GT-3, GT-13, and GT-15, assayed on rat erythrocytes at pH 6.0 and pH 8.0, increased with respect to dose, reaching 74–99% and 80–99% after a 1- and 2-hr incubation period, respectively. P30 produced 86–96% and 78–93% hemolysis on human erythrocytes at pH 6.0 and

TABLE I. Differential susceptibility of erythrocyte species and subcellular distribution of the specific hemolytic activity of 3 *Trichomonas vaginalis* strains.

Strain	Subcellular fraction	Specific hemolytic activity* (HU/mg/hr)			
		Human		Rat	
		pH 6.0	pH 8.0	pH 6.0	pH 8.0
GT-3	TTE	<0.25	<0.25	<0.25	1.25 ± 0.001
	P30	2.67 ± 0.19	2.24 ± 0.09	6.71 ± 0.33	3.81 ± 0.30
GT-13	TTE	<0.25	<0.25	<0.25	1.59 ± 0.39
	P30	4.08 ± 0.15	2.74 ± 0.15	10.81 ± 0.83	5.75 ± 0.65
GT-15	TTE	<0.25	<0.25	<0.25	2.14 ± 0.26
	P30	3.08 ± 0.00	2.81 ± 0.06	11.60 ± 0.24	5.54 ± 0.46

* Averages ± SE from 3 experiments, in triplicate, were plotted and the mean hemolytic dose (HD_{50}) interpolated. One hemolytic unit (HU) was defined as 1 HD_{50}, which was equivalent to 50% of hemolysis (100% hemolysis was equivalent to 0.8 ± 0.05 unit of optical density at 415 nm). Hemolysis having values <0.25 HU/mg/hr did not reach 50% hemolysis. Other values were significantly different from each other ($P < 0.05$).

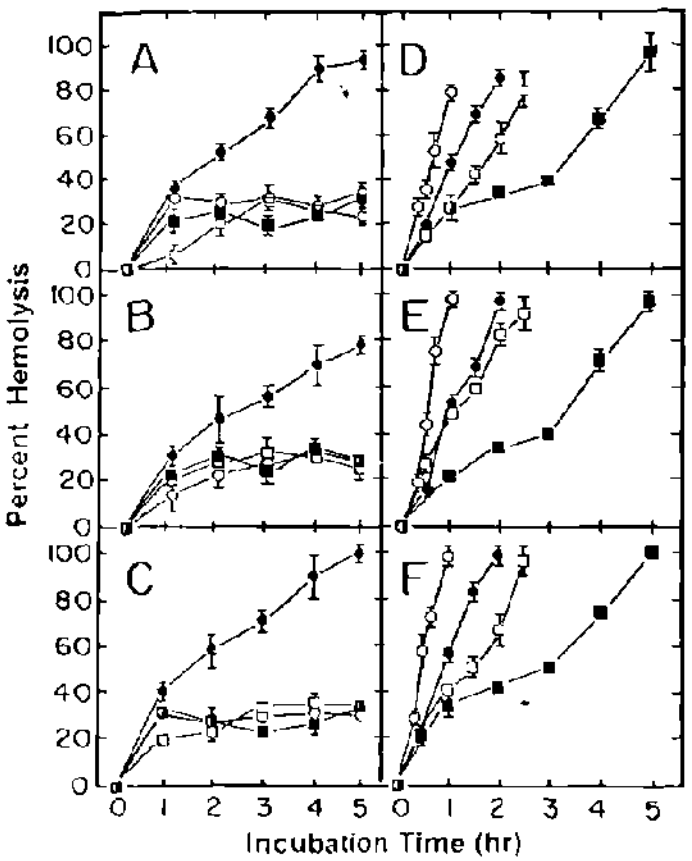

FIGURE 4. Time course of the hemolytic activity of trichomonad cell-free preparations. Frames A–C correspond to the hemolytic activity of GT-3, GT-13, and GT-15 of TTE, respectively. Frames D–F correspond to that of P30 from these same strains in the same order. The hemolysis mixtures were adjusted at pH 6.0 with rat (○) or human (□) erythrocytes added or at pH 8.0 with rat (●) or human (■) red blood cells added. All assays were performed at 36.5 C. Trichomonad total extracts and P30 assays contained 100 and 200 μg protein, respectively. Each point corresponds to the average ± SE of 3 determinations, in triplicate. Even though S30 from the aforementioned strains was tested, any hemolytic activity that was detected was not plotted.

pH 8.0 after 2.5 and 5 hr incubation, respectively. In all instances GT-3 showed the lowest hemolysis and GT-15 the highest. Hemolysis produced by TTE did not reach 50%, and S30 had no detectable activity.

Table I shows that the highest specific hemolytic activity corresponded to P30 from the GT-15 and GT-13 strains on rat erythrocytes at pH 6.0, with significant differences between each as in the case of P30 from GT-3 ($P < 0.005$) under the same experimental conditions. The specific hemolytic activity of the 3-strain P30 preparations on rat erythrocytes at pH 6.0 was 1.6–2.0 times higher than that at pH 8.0 ($P < 0.05$) and 2.5–3 and 3–4 times higher than the activity showed by this same preparation at pH 6.0 and pH 8.0 on human erythrocytes, respectively ($P < 0.05$).

Effect of Rosenthal's inhibitor on alkaline and acidic hemolytic activities

Figure 4 shows that the hemolytic activity of P30 assayed at pH 6.0 and pH 8.0 diminished as a function of Rosenthal's inhibitor. Under both experimental conditions, and in all the 3 trichomonad strains, 2 slopes were observed, one from 5 to 20 μM and another from 20 to 80 μM. The P30 hemolytic activity of GT-13 was more susceptible to the effect of Rosenthal's inhibitor at pH 8.0 than at pH 6.0 at all concentrations ($P < 0.05$). The dose of Rosenthal's inhibitor that produced 50% diminution at pH 6.0 and at pH 8.0 with respect to the untreated controls (ID_{50}) was 17.7 μM in all 3 strains at pH 8.0, and at pH 6.0 it was 18.8 μM, 16.6 μM, and 55.6 μM in GT-3, GT-15, and GT-13, respectively.

Alkaline and acidic specific PLA activities and effect of Rosenthal's inhibitor

The highest specific acidic and alkaline PLA activities corresponded to GT-15, followed by those of GT-13, these being 4% and 27% lower ($P < 0.005$ in both cases), and then by those of GT-3, which were 12% and 30% lower than those of GT-15, respectively ($P < 0.005$ in both cases) (Table II). Rosenthal's inhibitor (80 μM) diminished PLA acidic activity of GT-3, GT-13, and GT-15 by 27%, 36%, and 45%; and alkaline activity by 29%, 18%, and 54%, respectively ($P < 0.005$).

TABLE II. Inhibition of trichomonads specific PLA activity by Rosenthal's inhibitor.

	PLA specific activity (PLAU/mg/hr)*			
	Untreated controls		Added with Rosenthal's inhibitor	
Strain	Acidic†	Alkaline‡	Acidic†	Alkaline‡
GT-3	215 ± 40§	166 ± 20‖	157 ± 30#	118 ± 14¶
GT-13	235 ± 40§	174 ± 42‖	152 ± 25¶	90 ± 4¶
GT-15	244 ± 40	237 ± 4	134 ± 22¶	109 ± 9¶

* P30 preparations, having 200 μg of protein, were incubated for 1 hr at pH 6.0 or for 2 hr at pH 8.0 in the presence or absence of 80 μM Rosenthal's inhibitor. Specific PLA activity was expressed as Units of PLA (PLAu) per milligram of protein for 1 hr incubation at 36.5 C. Data correspond to averages ± SE of 3 experiments in triplicate.

† Assayed at pH 6.0.

‡ Assayed at pH 8.0.

§ $P < 0.005$ with respect to the acidic specific activity of GT-15.

‖ $P < 0.005$ with respect to the alkaline specific activity of GT-15.

$P = 0.11$ with respect to the acidic specific activity of GT-15.

¶ $P < 0.005$ with respect to the specific activity of preparations from the correspondent strain that were not added with Rosenthal's inhibitor.

Ultramicroscopic appearance of P30

When observed by transmission electron microscopy, P30 exhibited hydrogenosomes, which are characteristic for this species, in addition to vesicles and vacuoles.

DISCUSSION

In this study we have shown that hemolytic activity in rat and human erythrocytes TTE and P30 was time and dose dependent at pH 6.0 and pH 8.0. Rat blood cells were significantly more susceptible to hemolysis than were human erythrocytes.

The strikingly lower specific hemolytic activity of TTE than that of P30 at both pH values suggests that TTE possesses strong inhibitors and that both the alkaline and the acidic PLA-hemolysins of these parasites require activation. In our study this activation could have occurred during acquisition of P30, as it did with the hemolytic activity of *E. histolytica* (Said-Fernández and López-Revilla, 1982).

All trichomonad hemolytic activity was localized in P30, which is rich in membranes, hydrogenosomes, vesicles, and vacuoles. This result strongly suggests that trichomonad hemolytic factors are associated with the plasma membrane or vesicles inasmuch as hydrogenosomes and vacuoles have no hemolytic activity, as far as we know. Thus, hemolysins contained in P30 could contribute to contact-dependent cytolysis, as has been proposed previously (Gilbert et al., 2000).

Hemolytic activity of *T. vaginalis* was dependent on pH. All 3 strains tested in this study showed 2 peaks of maximum hemolytic activity, one at pH 6.0 and the other at pH 8.0. This indicates that trichomonads have both acidic and alkaline hemolysins. P30 from GT-3, GT-13, and GT-15 have both alkaline and acidic PLA activities, causing the hemolytic effect to be faster and stronger at pH 6.0 than at pH 8.0. In addition, trichomonad hemolytic activity showed a clear dependence on Ca^{2+}.

PLA enzymes showing strong hemolytic activity have been identified and characterized in diverse parasitic protozoa. These enzymes along with *T. vaginalis* hemolytic activity share their dependence on Ca^{2+} and pH (Opperdoes and VanRoy, 1982; Dennis, 1983; Said-Fernández and López-Revilla, 1988; Poirriez et al., 1995; Vargas-Villarreal et al., 1995, 1998). Trichomonad PLA activity assayed at the same pH values (6.0 and 8.0) in the presence of 1 mM Ca^{2+} was detected in all 3 trichomonad strains included in the current study. Specific hemolytic and PLA activities were lower in GT-3 than in GT-13 and GT-15 ($P < 0.05$). GT-3 is also less cytopathogenic than GT-13 and GT-15 (González-Robles et al., 1995). By analyzing the association of the specific acidic hemolytic activity with the specific acidic PLA activity and that of the specific alkaline hemolytic activity with the specific alkaline PLA activity of GT-3, GT-13, and GT-15, we found very high positive correlation coefficients ($r = 0.99$ in both cases). Thus, alkaline and acidic hemolytic activities and alkaline and acidic PLA activities in *T. vaginalis* are statistically related to each other and to the cytopathogenicity of this species.

We also analyzed the effect of Rosenthal's inhibitor on both specific hemolytic and specific PLA activities. In all the 3-strain preparations, and at both pH 6.0 and pH 8.0, PLA and hemolytic activities were blocked by Rosenthal's inhibitor. Nevertheless, under the same experimental conditions, PLA activity was less inhibited than the hemolytic effect. Rosenthal's inhibitor is an analog of phosphatidylcholine, a natural substrate of phospholipase enzymes (Rosenthal and Geyer, 1960), and is highly specific for PLA enzymes (Vargas-Villarreal et al., 1991). In our experience, Rosenthal's inhibitor is strikingly less hemolytic than another well-known PLA inhibitor, 1-11-carboxyundecyl-2-hexadecyl-L-1-glicero-3-phosphatidylcholine (Roch and Snyder, 1975). This strongly suggests that hemolytic activity is at least partially due to PLA enzymes. As noted above, Rosenthal's inhibitor produced a lower inhibition on PLA activity than that observed in hemolytic activity. This could be due to the fact that phospholipases are not direct cytolysins. In the first step of their hemolysis mechanism, phospholipases hydrolyze phospholipids from the cell surface, but hemolysis does not occur until a sufficient quantity of free fatty acids and phospholysoderivates (phospholipid hydrolysis products) have accumulated. These PLA products act as detergents on the cell membrane and stimulate and potentate PLA activity. Thus, the hemolytic effect of these enzymes is due, in fact, to the triple action of PLA activity and its 2 detergent products (Dennis, 1983), and blocking the enzyme inhibits the production of all 3 hemolysins. We postulate that the 27–54% inhibition of PLA activity, shown as the effect of Rosenthal's inhibitor, was enough to stop a critical quantity of free fatty acids and phospholysoderivatives from being accumulated, thereby blocking the hemolytic effect of PLA enzymes.

We did not observe 100% hemolysis inhibition under any experimental condition. This result could be due to the presence of other hemolysins working under the same experimental conditions as those used in this study or to the fact that Rosenthal's inhibitor is not 100% effective in blocking trichomonad PLA activity.

The remaining hemolytic activity in trichomonad preparations treated with Rosenthal's inhibitor is hardly attributable to the trichomonad pore-forming proteins described by Lynn et al. (1993, 1996), as long as Lynn and his colleagues observed the maximum hemolytic activity of pore forming protein at pH 5.6, whereas under our assay conditions, the maximum hemolytic activity occurred at pH 6.0 and pH 8.0. Moreover, in our study

the trichomonad hemolytic activity assayed at pH 5.6 was 25% lower than that at pH 6.0, and at pH 8.0 the hemolysis produced by the pore-forming protein was null. On the other hand, the effect of the pore-forming protein does not depend on Ca^{2+}, as was seen in hemolytic and PLA activities.

We conclude that *T. vaginalis* alkaline and acidic hemolytic activities depend, at least partly, on alkaline and acidic PLA, respectively. Alkaline and acidic PLA need to be isolated and further characterized to investigate whether these enzymes are relevant in the cytolytic mechanism of nucleated cells, particularly cells from vaginal mucosa. These PLA pore-forming proteins and perhaps other hemolysins could make up a cytolytic mechanism involved in the virulence of this species, as it does in several other parasitic protozoa.

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