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RESPUESTA INMUNE HUMORAL ESPECIFICA CONTRA PREPARACIONES DE PROTEINAS DE LA MEMBRANA EXTERNA DE *Salmonella typhi*

T E S I S

QUE PARA OBTENER EL GRADO ACADEMICO DE

DOCTOR EN BIOTECNOLOGIA

P R E S E N T A

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RESUMEN

Se diseñó un inmunoensayo enzimático (FT-ELISA) para la detección de anticuerpos séricos en pacientes con fiebre tifoidea (FT), utilizando preparaciones de proteínas de la membrana externa (pPME) de *Salmonella typhi*, como antígeno de captura.

De un total de 270 sueros evaluados con el FT-ELISA, obtenidos de individuos de dos regiones geográficas endémicas para FT, México y Malasia, se detectaron 66 sueros positivos, de los cuales 55 fueron confirmados por hemocultivo y 9 por Prueba de Widal, mientras que existió un falso positivo (fiebre paratifoidea) así como un positivo sin prueba confirmatoria. En relación a los 191 sueros controles, se incluyeron 43 de donadores de sangre clínicamente sanos y 148 de pacientes con una amplia gama de infecciones, tanto septicémicas como localizadas, causadas por bacterias Gram-negativas y Gram-positivas, por virus y por rickettsia, principalmente. De estos sueros controles, únicamente se detectaron dos probable falsos negativos.

El FT-ELISA fue eficiente para discriminar entre pacientes con FT (síntomas presentes entre menos de una semana y un mes) y pacientes con otras infecciones. Al mismo tiempo mostró ser una prueba alternativa al hemocultivo, con las ventajas de utilizar una cantidad menor a 0.5 ml (a una dilución única de 1:3,125) contra 15-30 ml; así como resultados en 4.5 en vez de 72 horas. Con los resultados obtenidos, se sugiere que el ensayo podría ser un buen candidato para el diagnóstico rutinario de la FT.

Las pPME utilizadas como antígeno contenían 25-30% de lipopolisacárido por peso, y, mediante ensayos de inhibición en ELISA, con pPME o LPS como antígenos de captura y como antígenos libres, se demostró que tanto las proteínas como el LPS juegan un papel importante en la detección de anticuerpos inducidos durante la infección por *S. typhi*. Con base en la literatura internacional y en nuestros resultados, podemos decir que las PME y el LPS podrían estar formando epítopes complejos, o que dichos complejos son necesarios para mantener inalterada a la proteína nativa o a la estructura del LPS.

Por otra parte, se utilizaron dos péptidos como antígeno en ELISA, OmpC-1 y OmpC-2, los cuales corresponden a dos regiones *in/del* de la porina OmpC de *S. typhi* con respecto a su homóloga en *E. coli*. Se evaluaron dos grupos de sueros, uno de pacientes con FT y otro de controles, y con los resultados obtenidos se observó una heterogeneidad. Sin embargo, el uso de estos péptidos fue importante para explorar, de manera puntual, la respuesta inmune humoral contra moléculas pequeñas y sintéticas, que no necesariamente se van a conformar como lo harían en su entorno natural. Así mismo, se pudo observar que la respuesta inmune humoral individual es diferente, pudiendo depender parcialmente del estado inmunológico de la personas, así como de su idiosincrasia genética.

En conclusión, la aportación de este trabajo fue el diseño de un nuevo sistema para la detección rápida y precisa de la FT, que se debe continuar validando en diferentes laboratorios del mundo. Conceptualmente, podemos decir que la respuesta inmune humoral sérica específica en contra de las PME, depende de conformaciones e interacciones que todavía deben ser elucidadas al detalle.

SUMMARY

An enzyme-linked immunosorbent assay (TF-ELISA) was designed to detect seric antibodies in typhoid fever (TF) patients, using *Salmonella typhi* outer membrane protein preparations (OMPp) as the capture antigen.

From 270 evaluated sera with the TF-ELISA, obtained from individuals of two endemic regions for TF, Mexico and Malaysia, 66 positive sera were detected; from these, 55 were confirmed by blood culture and nine by the Widal Test, whereas there was one false positive (paratyphoid fever) and one serum without its confirmatory test. In relation to 191 control sera, 43 were from healthy blood donors and 148 from patients with several kinds of infections, either septicemic or localized, caused by Gram-negative and Gram-positive bacteria, and viruses and rickettsia. From this control sera, only two probable false negatives were detected.

The TF-ELISA was efficient to discriminate between TF patients (having less than a week to one month with clinical symptoms) and patients with other infections. Also, this test, proved to be an alternative to blood culture, having advantages such as the need of less than 0.5 ml of blood (for use at a single serum dilution of 1:3,125) instead of 15-30 ml, and the the obtention of results in 4.5 instead of 72 hours. Therefore, we suggest that the assay could be a good candidate for the routinary diagnosis of TF.

OMP preparations used as antigen had a 25-30% lipopolysaccharide (LPS) content by weight, and, by inhibition ELISA, using OMPp or LPS as capture and free antigens, it was demonstrated that both kinds of antigen play an important role in the detection of antibodies induced by *S. typhi*. Based on the results obtained in other laboratories and on our results, we can say that OMPp together with LPS could be forming complex epitopes, or maybe that such complexes are necessary to maintain unaltered native protein or LPS structures.

On the other hand, two peptides, OmpC-1 and OmpC-2, were used as antigen in the TF-ELISA. Both peptides correspond to *in/del* regions from *S. typhi* OmpC porin as compared with its *E. coli* homologue. Two groups of sera, from TF patients and controls, were tested, and we observed a heterogeneity in the results obtained. However, the use of these peptides was important to explore, in a specific way, the humoral immune response against small and synthetic molecules, that maybe do not adopt their natural conformation. Also, variations in the individual humoral immune response were observed, which could depend on the personal immunologic status as well as on their genetic idiosyncrasy.

In conclusion, the contribution of this study was the design of a new system for the rapid and precise detection of TF, that must be continually validated in different laboratories of the world. Conceptually, we can say that the seric specific humoral immune response against OMPp, depends on conformations and the interactions that must be elucidated in detail.

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A. Verdugo-Rodríguez, Y. López-Vidal, J. L. Puente, G. M. Ruíz-Palacios and E. Calva.

European Journal of Clinical Microbiology & Infectious Diseases (1993) 12(4):

-Apéndice II:

"Detection of antibodies against *Salmonella typhi* outer membrane protein (OMP) preparations in typhoid fever patients"

A. Verdugo-Rodríguez, L.-H. Gam, S. Devi, S. D. Puthucheary, E. Calva and T. Pang.

Sometido a: *Asian Pacific Journal of Allergy and Immunology*.

-Apéndice III:

"*Salmonella typhi* outer membrane proteins in the diagnosis of typhoid fever"

A. Verdugo-Rodríguez, Y. López-Vidal, J. L. Puente, F. J. Santana, G. M. Ruíz-Palacios and E. Calva

In: *TYPHOID FEVER: Strategies for the 90's*. Ed. T. Pang, C. L. Koh and S. D. Puthucheary. World Scientific, Singapore, 1992, pp.: 216-220.

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J. L. Puente, A. Verdugo-Rodríguez and E. Calva.

Molecular Microbiology (1991) 5(5):1205-1210.

VI. LITERATURA CITADA

INTRODUCCION

Salmonella typhi es un bacilo Gram-negativo, que infecta específicamente al humano ocasionando una bacteremia denominada fiebre tifoidea (FT).

En 1986, se reportaban anualmente, 12.5 millones de casos de FT en el mundo, excluyendo China, con una tasa de mortalidad del 1% (Edelman y Levine, 1986). Desafortunadamente, como sucede con la mayoría de las infecciones gastroentéricas, la incidencia mas alta de FT se presenta en países del tercer mundo, en los cuales, el desarrollo de tecnologías para diagnosticar, controlar, y erradicar enfermedades infecto-contagiosas se ve limitado por la carencia de recursos económicos y humanos.

Otro de los aspectos por los cuales el conocimiento acerca de *S. typhi* y sus mecanismos de patogenia ha sido limitado, se debe a que la bacteria es específica de huésped, es decir, no se ha encontrado modelo animal alguno que se infecte de manera natural. Al respecto, los grupos de investigación interesados en el tema, han estudiado el modelo "*S. typhimurium*-ratón", así como ensayos *in vitro*, para modelar la patogénesis de la FT en el humano (Calva et al., 1988).

S. typhi penetra al organismo mediante la ingestión de agua o alimentos contaminados con heces fecales, es decir, vía oral-fecal. Se ha propuesto que las bacterias del género *Salmonella* se adhieren y penetran al epitelio intestinal, a través de las células "M" de las placas de Peyer, donde son ingeridas por los macrófagos (sin destruirlas) y transportadas al sistema retículo endotelial, para multiplicarse y propagarse por todo el organismo, colonizando principalmente los nódulos linfáticos, el bazo y el hígado (Finlay y Falkow, 1989). Se ha descrito que la multiplicación de *Salmonella* es intracelular, sin embargo, existe una propuesta antagónica, ya que Hsu (1989) ha demostrado que la replicación extracelular de *S. typhimurium* en el ratón, parece ser prominente.

S. typhi, como todas las células bacterianas Gram-negativas, está cubierta por una membrana externa, la cual protege a dichas células de agentes nocivos deteniendo su penetración. Al mismo tiempo, los nutrimentos y los productos de desecho deben atravesar esta membrana velozmente, y ésto es posible mediante la presencia de varias proteínas de transporte (Nikaido y Vaara, 1985). Estas proteínas pueden dividirse en tres clases: no específicas o porinas generales; canales específicos; y sistemas de transporte dependientes de energía, de alta afinidad (Nikaido, 1992).

De éstas, las porinas OmpC y OmpF, que se conforman triméricamente, así como OmpA (una proteína estructural monomérica), son las más abundantes en la membrana externa, presentándose cada una en aproximadamente 10^5 moléculas por célula. Así mismo, se han observado más de veinte proteínas menores, de las cuales algunas se sobreexpresan bajo condiciones ambientales específicas. Otro componente principal de la membrana externa es el lipopolisacárido (LPS), que constituye aproximadamente el 45% de la cubierta celular (Lim, 1986). Uno de los componentes del LPS es el denominado antígeno "O", constituido por cadenas repetidas de azúcares.

Otros elementos externos predominantes en la bacteria son los flagelos, los cuales son perítricos en el caso de *S. typhi*, y se conforman de unidades de flagelina constituyendo el antígeno "H"; así como una cápsula incompleta polisacarídica, denominada antígeno "Vi".

El objetivo principal de nuestro laboratorio ha sido el estudio de la relación estructura-función de las proteínas de la membrana externa (PME) de *S.typhi* y de sus respectivos genes. Al respecto, previamente se clonaron los genes *ompC* (Puente et al., 1987); *ompF* (Calva et al., 1992)); y *phoE* (Puente, 1987; Torres, 1993). Al mismo tiempo, nos hemos enfocado en la utilización, ya sea de secuencias de DNA o de sus productos proteicos, como posibles herramientas para la identificación (directa o indirecta) de manera específica de *S. typhi*. Este enfoque se debe al hecho de que el diagnóstico eficiente de la FT representa un serio problema en la actualidad. En este sentido, diferentes grupos han abordado el tema con formas y expectativas variables, habiendo utilizado antígenos como LPS, Vi, extractos proteicos, PME y flagelina; anticuerpos monoclonales; sondas de DNA, entre otros (Beasley et al., 1981; Tsang et al., 1981; Barrett et al., 1983; Taylor et al., 1983; Nardiello et al., 1984; Calderón et al., 1986; Srivastava and Srivastava, 1986; Appasakij et al., 1987; Tsang and Chau, 1987; Rubin et al., 1989; Sippel et al., 1989; Rubin et al., 1990; Sadallah et al, 1990; Choi et al., 1992; Ismail et al., 1992).

Con respecto a las PME y su papel en la respuesta inmune humoral, diversos grupos han demostrado que los anticuerpos séricos de pacientes con FT son capaces de reconocer dichas proteínas (Beasley et al., 1981; Calderón et al., 1986). Así mismo, se ha observado que las PME de *S. typhi* y de *S. typhimurium*, en el modelo del ratón,

son capaces de inducir una respuesta inmune protectora (Isibasi et al., 1988; Udhayakumar and Muthukkarupan, 1987).

Al mismo tiempo, se han demostrado diferentes papeles de algunas PME (porinas) en el proceso de patogenia, tales como efectos inflamatorios, en la liberación de citocinas, activación de complemento, daño renal y cardiovascular, entre otros (Galdiero et al., 1984, 1986, 1990, 1993; Tufano et al., 1987, 1989). Es importante señalar que, tradicionalmente, estos efectos se han adjudicado a la presencia del LPS.

En nuestro laboratorio, en colaboración con el Departamento de Infectología del Instituto Nacional de la Nutrición "Salvador Zubirán", se diseñó un inmunoensayo enzimático "ELISA" ("enzyme-linked immunosorbent assay"), utilizando preparaciones de PME (pPME) de *S. typhi* como antígeno, con la finalidad de detectar anticuerpos específicos contra dichas pPME, en sueros de pacientes con FT. Al mismo tiempo, dado que las pPME utilizadas contenían 30% de LPS, nos preguntamos acerca de la participación de los dos componentes de este antígeno en la respuesta inmune humoral a detectar.

Previamente, a través de ensayos tanto *in vitro* como *in vivo*, se ha demostrado en diferentes bacterias Gram-negativas que el LPS juega un papel importante en resistencia al suero, a lisis por complemento, en invasión y lisis celular, entre otros eventos asociados a virulencia (Okamura et al., 1983; Engels et al., 1985; Porat et al., 1987; Mäkelä et al., 1988; Mroczenski-Wildey et al., 1989; Al-Hendy et al., 1992). Así mismo, se ha demostrado que el LPS es capaz de inducir inmunidad protectora, aunque ésta puede ser inespecífica o parcial (Isibasi et al., 1988; Vuopio-Varkila et al., 1988).

Con respecto a la interacción del LPS con PME, Saxén et al., (1986), y Udhayakumar y Muthukaruppan (1987), demostraron que la presencia del LPS, asociado a una porina, es indispensable para la inmunidad protectora generada en contra de *S. typhimurium* en el ratón. Así mismo, Sen y Nikaido (1991), han reportado que células productoras de LPS incompleto (fenotipo "altamente rugoso") no pueden ensamblar niveles normales de proteínas, especialmente porinas, en la membrana externa. Dichas moléculas de LPS defectuoso fueron incapaces de facilitar la inserción y trimerización de la porina OmpF *in vitro*. Un problema de trimerización similar fue reportado con monómeros de PhoE (Cock et al., 1990).

Por otra parte, como se mencionó anteriormente, Puente et al., en 1987, reportaron la clonación del gene que codifica para la PME OmpC de *S. typhi*, la cual no se osmoregula [**Apéndice IV** (Puente et al., 1991)], en contraste con OmpC de *E. coli*, que se expresa preferencialmente cuando la bacteria es cultivada en condiciones de alta osmolaridad (20% de sacarosa), y se reprime cuando la bacteria es cultivada en condiciones de baja osmolaridad (Kawaji et al., 1979). Así mismo, a diferencia de OmpC de *E. coli*, OmpC de *S. typhi* no es aparentemente regulada por temperatura. Con los datos anteriores, se puede pensar que la presencia de esta proteína en la membrana externa es relevante para la bacteria, por lo cual se ha decidido explorar algunos aspectos relacionados con la respuesta humoral.

Cuando OmpC de *S. typhi* se caracterizó y comparó con su homólogo en *Escherichia coli* (Puente et al., 1989), se determinó que era similar en un 79%. Al compararse sus correspondientes secuencias de aminoácidos (aa), se detectaron dos regiones hipervariables, correspondiendo a regiones *in/del* en *S.typhi* con respecto a *E. coli*. La primera de ellas, altamente hidrofílica, está situada entre los aa 152 a 167, mientras que la segunda, mas hidrofóbica que la primera, se sitúa entre los aa 245 y 259. Con la finalidad de conocer la respuesta inmune humoral hacia estas dos regiones variables, a través de inmunoensayos, se diseñaron dos péptidos de las regiones hipervariables mencionadas, 150-169 y 243-262. Los inmunoensayos se realizaron con sueros de pacientes con FT.

Con la finalidad de estudiar la topología de la porina OmpC, Puente et al., (1992) y Juárez (1993), insertaron entre los aa 160-161 y 252-253, respectivamente, un oligonucleótido correspondiente a una parte de la secuencia que codifica para la proteína VP4 de rotavirus que, al ser expresado, fue detectado por un anticuerpo monoclonal neutralizante, en ELISA, tanto en pPME obtenidas de *E. coli* conteniendo el plásmido con *ompC* quimérico, como en células completas. Asimismo, se observó lo anterior mediante microscopía electrónica, utilizando marcaje con oro coloidal. Con estos resultados se determinó que el epítope detectado es expuesto al exterior de la célula, aunque dicha detección fue 60% mayor cuando la inserción se realizó en la región altamente hidrofílica (la región de 160-161).

MATERIALES Y METODOS

Cepas bacterianas

1. *Salmonella typhi* IMSS-1 (Ver **Apéndices I y II**).
2. *S. typhi* 100 (Ver **Apéndice II**).
3. *S. typhi* Ty-403 j-z66, cepa procedente de Indonesia (3483/82 Le Minor).
4. *S. typhi* Ty-404 d-z66, cepa procedente de Indonesia (3083/80 Le Minor).

   Las cepas 3 y 4 tienen el antígeno H llamado z-66 por Le Minor, que las hace tener una movilidad diferente a la de *S. typhi* con el antígeno H (d) (Le Minor et al., 1981). Estas cepas fueron proporcionadas amablemente por el Dr. Gary Schoolnik de la Universidad de Stanford, EUA.
5. *S. typhi* Ty2, D9, 12d, Vi, cepa de referencia de la *American Type Culture Collection* (*ATCC*).
6. *S. typhimurium* LT2, cepa original de Herzberg obtenida a través del Dr. Bruce Stocker de la Universidad de Stanford, EUA.
7. *S. arizonae*, aislado de líquido articular de una paciente con artritis séptica. Caracterizada bioquímicamente; serológicamente, solo aglutinó con el antisuero polivalente de *Salmonella*, de los grupos A hasta el I, además de Vi (serobac antisuero S-1, Bigaux Diagnóstica).
8. *S. worthington* serotipo G2 (1, 13, 23, z), aislamiento clínico de un brote de gastroenteritis en Mérida, Yuc.
9. *S. enteritidis* serotipo D1 (1, 9, 12)), aislamiento de un paciente con gastroenteritis.
10. *S. aberdeen* serotipo F (11, i, 1, 2), cepa de referencia (Bigaux Diagnóstica).
11. *S. anatum* serotipo E1 (3, 10e, h1, 6), cepa de referencia (Bigaux Diagnóstica).
12. *S. paratyphi* serotipo A (1, 2, 12), cepa de referencia (Bigaux Diagnóstica).
13. *S. paratyphi* serotipo B (1, 4, [5], 12), cepa de referencia (Bigaux Diagnóstica).

    Las cepas núm. 7-13 fueron proporcionadas amablemente por la M. en B. Miriam Bobadilla, del Laboratorio de Microbiología Clínica del Depto. de Infectología, Instituto Nacional de la Nutrición "Salvador Zubirán".

Preparaciones de PME.

Se describe en **Materiales y Métodos** de los **Apéndices I, II y III.**

Preparaciones de LPS.

Doscientos ml de un cultivo de siete hr, suspendidos en 100 ml de agua deionizada, fueron esterilizados en el autoclave durante 30 min, centrifugados, y la pastilla resultante fue diluida en PBS pH 7.4, SDS 0.5%. Proteinasa K (400 µg/ml) (Proteasa tipo XXVIII, Sigma P4914) se agregó a esta suspensión, la cual fue incubada toda la noche a 65°C. Posteriormente se centrifugó 3 hr a 40,000 rpm a temperatura ambiente, en un rotor Beckman 55.2 Ti; la pastilla resultante fue lavada en PBS y centrifugada dos veces. Finalmente, a la pastilla resultante, diluida en PBS, se le denominó preparación de LPS (LPSp). Al LPS comercial (Sigma L2387) se le denominó LPS sigma (LPSs). La cuantificación del LPS, utilizando LPSs como estándar, se realizó midiendo el contenido de 2-*keto*-3-*deoxyoctonate* (KDO), como se describió previamente (Karkhanis et al., 1978).

Sueros.

Un esquema de inmunización con pPME de *S. typhi*, *S.typhimurium*, *S. aberdeen* y *S. anatum*, fue llevado a cabo en ratas, con la finalidad de conocer la respuesta de anticuerpos contra las pPME en un modelo animal. Grupos de cuatro ratas Wistar, sexo masculino, de tres meses de edad, fueron inmunizados mediante una inyección inicial, subcutánea, con 20 µg de pPME en adyuvante completo de Freund (Gibco Laboratories, Grand Island, N.Y., USA); seguida de una inmunización a las dos semanas, y posteriormente de dos inmunizaciones semanales, intravenosas, con 20 µg de pPME sin adyuvante. Los animales fueron sangrados una semana después de cada inmunización. Los sueros fueron colectados y congelados a -70°C hasta su utilización.

ELISA con pPME de distintas Salmonella y de E. coli

Un aspecto relevante en la validación de la utilidad de los resultados obtenidos con el FT-ELISA (**Apéndices I y II**), es la capacidad del sistema para discriminar entre los sueros de pacientes con FT y de controles infectados con diferentes especies de *Salmonella*. Debido a que se carecía de estos sueros, se planteó la realización de ELISAs, con pPME de distintas especies de *Salmonella* como antígenos, utilizando algunos sueros de pacientes positivos a FT, así como controles.

Inhibición en ELISA

Los ensayos de inhibición en ELISA con los antisueros de rata, se llevaron a cabo como se describe en el **Apéndice I** en **Materiales y Métodos**. Los antisueros fueron titulados en ELISA, contra pPME y contra LPSp, y posteriormente utilizados en inhibición en ELISA con su título concentrado cinco veces. Es decir, los títulos para pPME fueron 1/3,125 y se utilizaron a 1/625, mientras que para LPS los títulos fueron 1/625, y se utilizaron a 1/125.

Péptidos

Con la finalidad de explorar la respuesta inmune humoral hacia dos regiones *in/del* de OmpC de *S. typhi* con respecto a la OmpC de *E. coli*, se diseñaron dos péptidos de las regiones 150-169 y 243-262 de la proteína, altamente hidrofílico e hidrofóbico, respectivamente. A continuación se describen las secuencias correspondientes, habiéndose basado en la secuencia reportada por Puente et al., (1989).

Péptido OmpC-1: Correspondiente a la región comprendida entre los aminoácidos 150 a 169. Expuesta en la membrana. Altamente hidrofílica entre los asteriscos.

150 160 170

* *

Q G K N G S V S G E E N T N G R S L L N Q

Péptido OmpC-2: Correspondiente a la región comprendida entre los aminoácidos 243 a 262. Probablemente no expuesta en la membrana. Mayor hidrofobicidad entre los asteriscos.

243 250 260

* *

T R F G T S N G S N P S T S Y G F A N K

1) Síntesis química de los péptidos

Los péptidos utilizados en este trabajo fueron sintetizados por el M. en C. Fernando Zamudio en el laboratorio del Dr. Lourival Possani, siguiendo el método descrito por Merrifield, para la síntesis química de péptidos en fase sólida (Merrifield et al., 1963; Mitchell et al., 1978). Todos los N-ter-butiloxicarbonil aminoácidos (Boc-aminoácidos) y los ter-Boc-aminoácidos-Resinas (poliestireno derivatizado con grupos químicos reactivos aminados o halogenados) fueron adquiridos de Peninsula Corporation (California, USA).

2) Acoplamiento

Las reacciones de acoplamiento fueron llevadas a cabo con tres excesos molares de t-Boc-aminoácido y diciclohexilcarbodiimida (DCC), ésta última como agente acoplante; hidroxibenzotriazol como agente antirracemizante; diclorometano, 2-propanol y dimetilformamida como solventes.

3) Desprotección

La remoción de los grupos protectores (t-Boc) de los aminoácidos se llevó a cabo con ácido trifluoroacético al 50% en diclorometano, por espacio de 30 min en agitación.

Cada uno de los pasos de desprotección y acoplamiento fueron monitoreados por medio de la prueba de ninhidrina (Sarin et al., 1984), y el ciclo de acoplamiento se repetía hasta que la prueba de la ninhidrina fuera negativa.

Los péptidos sintetizados fueron separados de la resina por tratamiento drástico con ácido fluorhídrico (HF)(10 ml de HF/g de péptido-resina) por 30 min a 4°C usando anisol (1 ml/g de péptido-resina). Posterior a la ruptura, la mezcla de péptido-resina fue lavada con 30 ml de eter, y finalmente el péptido fué extraído con ácido acético al 5%. El material obtenido fue liofilizado y guardado a -20°C hasta su utilización.

4) Purificación de los péptidos

Los péptidos fueron purificados por cromatografía, a través de una columna de sephadex G-10 (Pharmacia, Uppsala, Sweden) equilibrando con agua.

Péptidos en ELISA

Los péptidos OmpC-1 y OmpC-2, fueron utilizados como antígeno unido a la placa, en una concentración de 50 µg/ml, los sueros se usaron a una dilución

recíproca de 200. El segundo anticuerpo, fue de conejo anti-humano acoplado a peroxidasa (DAKO). Los tiempos de reacción utilizados son aquellos que se describen para el FT-ELISA, a excepción del tiempo de reacción enzima-sustrato, el cual fue de ocho min.

RESULTADOS

FT-ELISA

a) pPME de *S. typhi.*

En los **Apéndices I y II** se muestran las **Figuras núm. 2** correspondientes, donde se puede observar que con el FT-ELISA (pPME de *S. typhi* como antígeno) se discriminó entre los sueros de pacientes con FT, desde la primer semana (**Apéndices I y II**) hasta un mes (**Apéndice II**) del inicio de la sintomatología clínica, con respecto a los sueros de pacientes con diferentes tipos de infecciones. Además de los sueros probados y graficados como se menciona arriba, se evaluaron otros sueros. En la **Tabla 1** (pags. 12 y 13) se enlistan todos los sueros probados.

Tabla 1

Resumen de los resultados obtenidos con el FT-ELISA

infección	origen	positivos/ probados	% de positividad
1) FT; HC(+)	Cd. México	15/15	100
2) FT; HC(+), W(+)	Malasia	40/40	100
3) sospechosos FT; HC(-), W(+)	Malasia	9/11	81.8
4) sospechosos FT; HC(-), W(-)	Malasia	1/48	2.1
5) donadores sanos	Cd. México	0/15	0
6) donadores sanos	Malasia	0/28	0
7) dengue (Arbovirus)	Malasia	0/9	0
8) leptospirosis (*Leptospira*)	Malasia	0/17	0
9) typhus (*Rickettsia*)	Malasia	0/19	0
10) *ETEC*	EUA	0/15	0
11) *Campylobacter jejuni*	Cd. México	0/15	0
12) Otras Salmonelas:			10
Salmonella grupo B	Chiapas	0/6	
S. enteritidis	Cd. México	0/1	
S. paratyphi B	Cd. México	0/1	
Salmonella spp.	Cd. México	0/1	
S. paratyphi A	Malasia	1/1	

Tabla 1 (continuación)

Resumen de los resultados obtenidos con el FT-ELISA

infección	origen	positivos/ probados	% de positividad
13) Otros:			0
E. coli	Cd. México	0/5	
Proteus spp.	Cd. México	0/2	
Shigella flexnerii	Cd. México	0/1	
Pseudomonas aeruginosa	Cd. México	0/1	
Staphylococcus epidermidis	Cd. México	0/1	
Staphylococcus coagulasa(+)	Cd. México	0/2	
Staphylococcus coagulasa(-)	Cd. México	0/1	
Enterococcus grupo D	Cd. México	0/1	
Streptococcus grupo B	Cd. México	0/1	
Acinetobacter coalcoaceticus	Cd. México	0/1	
Candida albicans	Cd. México	0/1	

Abreviaciones:

FT. Fiebre tifoidea

HC. Hemocultivo

W. Prueba de Widal

b) LPS de *S. typhi*.

El papel de los anticuerpos contra el LPS en la infección por *S. typhi* es importante, como se observó en ELISA utilizando como antígeno dos diferentes preparaciones de LPS de *S. typhi*, donde se probaron algunos sueros de pacientes con FT. Sin embargo, cuando se utilizó el LPSp (10-15% de proteína), fue necesaria una concentración de LPS 10 veces mayor que la contenida en las pPME, para poder detectar una respuesta similar en ELISA con pPME. Así mismo, cuando el LPSp fue suspendido en PBS/B-1 (solución reductora) y hervido durante 5 min, la varianza de los valores de absorbancia entre grupos de pacientes FT-positivos y controles se incrementó de manera similar a la observada con las pPME. Con respecto al LPSs (menos de 10 ng de proteína/µg de LPS), fue necesario utilizar una concentración de LPS 20 veces mayor, que aquella contenida en las pPME.

c) pPME de otras *Salmonella* y de *E. coli*

Una de las preguntas importantes en la validación del FT-ELISA, se refiere a la respuesta inmune que generen infecciones por otra clase de *Salmonella* diferentes a *S. typhi*. En vista de que se carecía de los sueros, se decidió explorar la alternativa de utilizar los sueros de pacientes con FT contra las pPME de algunas salmonelas y de *E. coli* como antígeno en ELISA. En la **Tabla 2** (pag. 15) se resumen los resultados obtenidos. Como se puede observar, la respuesta detectada varió dependiendo de la especie utilizada. En algunos casos la reactividad cruzada se puede considerar importante (mayor del 50%): *S. typhimurium*, *S. paratyphi* B y *S. arizonae*, mientras que en otros, nula o casi nula: *S. worthington*, *S. aberdeen* y *E. coli*.

Tabla 2

Porcentaje de positividad de sueros de pacientes con FT, positivos por hemocultivo, en ELISA con pPME de diferentes bacterias como antígeno de captura.

Bacteria	num. de sueros probados	num. de sueros positivos	% de positividad
S. typhi IMSS-1	19	19	100
S. typhi Ty-403	19	19	100
S. typhimurium	19	14	73.6
S. paratyphi A	19	4	21.0
S. paratyphi B	19	11	57.8
S. anatum (grupo E)	19	5	26.3
S. aberdeen (grupo F)	19	1	5.2
S. worthington (gpo.G)	19	0	0.0
S. arizonae	19	10	52.6
E. coli	16	0	0.0

Inhibición en ELISA

Considerando que las pPME utilizadas como antígeno en ELISA contienen 30% de LPS, nos preguntamos acerca de la participación de sus dos componentes (proteínas y LPS) en la respuesta inmune humoral detectada. Para este efecto, se diseñó el ensayo de inhibición en ELISA descrito en **MATERIALES Y METODOS** del **Apéndice I**, y de este escrito.

a) Sueros de humano con FT.

Los resultados obtenidos al utilizar pPME (5 µg/ml) como antígeno acoplado a la fase sólida, se muestran en la **Figura 3** del **Apéndice I**, donde se observó que 9 µg/ml de pPME solubles fueron suficientes para inhibir 50% ó mas de respuesta de anticuerpos de humano contra las pPME en la fase sólida, mientras que con LPSs soluble no se logró inhibir 50% de la respuesta, aún con 25 µg/ml. Así mismo, en la **Figura 1** (pag. 17), se muestra una gráfica con los resultados observados cuando se utilizó LPSs (35 µg/ml), acoplado a la fase sólida, donde 5 µg/ml de pPME solubles fueron capaces de inhibir mas del 50% de la respuesta, mientras que con 25 µg/ml de LPSs, únicamente se inhibió el 25%.

b) Sueros hiperinmunes de rata contra pPME.

Con la finalidad de explorar la respuesta de anticuerpos contra pPME de diferentes salmonelas en un modelo animal, como se mencionó en **MATERIALES y METODOS**, se emplearon sueros hiperinmunes anti-pPME de *S. typhi*, *S. typhimurium*, *S. anatum*, y *S. aberdeen*. La capacidad de las pPME o de LPSp (antígeno libre) para inhibir la unión de anticuerpos contra pPME, de cepas homólogas, se muestra en la **Figura 2** (pag. 18). Para tres cepas, *S. typhimurium* (a), *S. typhi* (b), y *S. aberdeen* (d), se detectó un 50% de inhibición de la unión de anticuerpos al antígeno unido a la fase sólida con 12-13 µg/ml de antígeno homólogo (pPME); mientras que con 25 µg/ml de LPSp la inhibición fue únicamente de 12 a 20%. La excepción fue con *S. anatum* (c), donde 25 µg/ml de pPME inhibieron 50% de la unión de anticuerpos, y la misma cantidad de LPSp inhibió únicamente el 25%.

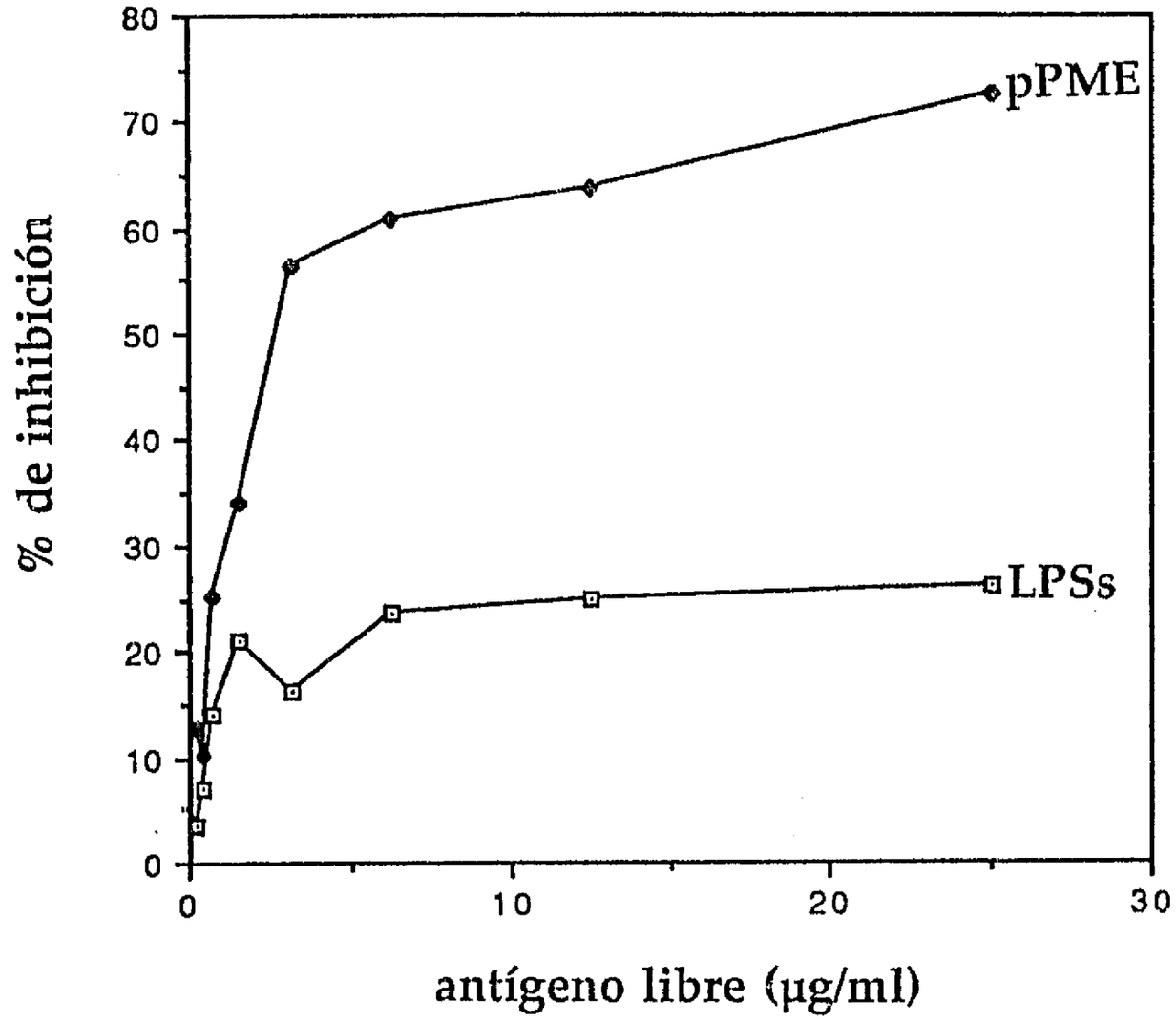

Figura 1. Inhibición en LPSs-ELISA. Inhibición de la unión de anticuerpos séricos de pacientes con FT, a LPSs acoplado a la fase sólida, por diferentes concentraciones de LPSs y de pPME de *Salmonella typhi*. El intervalo de concentración de los inhibidores probados se fue incrementando por duplicado.

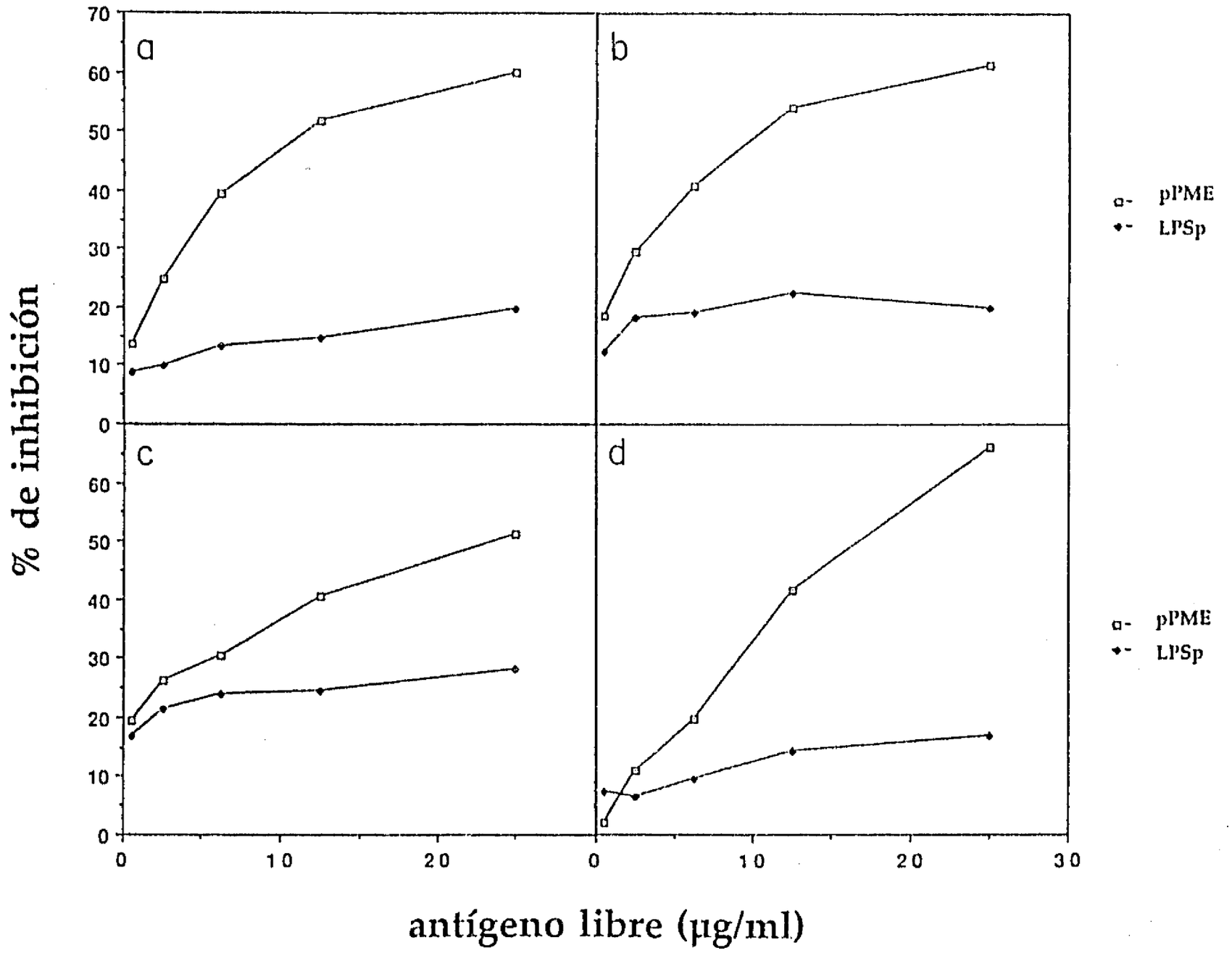

Figura 2. Inhibición de anticuerpos de rata contra pPME de *Salmonella* en ELISA. Inhibición de la unión de anticuerpos séricos de rata (diluído 1:125) a pPME acopladas a la fase sólida, por diferentes concentraciones de preparaciones homólogas de PME o de LPS, obtenidas de *S. typhimurium* (a), *S. typhi* (b), *S. aberdeen* (c), y *S. anatum* (d).

Péptidos en ELISA.

Una de las preguntas que surgieron, cuando se analizó la secuencia de aminoácidos de la proteína OmpC de *S. typhi*, fue la concerniente a la respuesta inmune humoral que podría estarse generando en pacientes con FT, contra dos posibles regiones variables expuestas hacia el exterior de la membrana externa, con característica particular de corresponder a dos regiones in/del, específicas de *S. typhi* con respecto a *E. coli*. Como se mencionó en **MATERIAL Y METODOS**, se diseñaron los péptidos OmpC-1 y OmpC-2, para utilizarse como antígenos en ELISA.

Con los resultados obtenidos (**Tabla 3**, pag. 20) se pudo observar que algunos de los sueros de los pacientes con FT reconocieron específicamente a los péptidos, otros sueros lo hicieron de manera intermedia, mientras que otros no los reconocieron. En resumen, no se pudo establecer una correlación con la respuesta detectada cuando se utilizaron las pPME como antígeno. Una observación similar se hizo con los sueros controles, es decir, mientras el valor de absorbancia de algunos sueros era basal, en otros casos era cercano al punto de corte. Así mismo, se observó en mas de la mitad de los ensayos, que cuando el suero de un paciente reaccionaba fuertemente contra el péptido OmpC-1, lo hacía contra el péptido OmpC-2

Todos los ensayos se realizaron por duplicado y presentaron una desviación estándar menor al 10%.

Tabla 3

Valores de absorbancia obtenidos en ELISA con los péptidos OmpC-1 y OmpC-2 como antígeno de captura.

	Antígenos	
pPME	**OmpC-1**	**OmpC-2**
Sueros positivos en FT-ELISA		
1.60	1.25	1.23
1.70	1.25	1.14
1.63	0.81	0.87
1.62	0.40	0.42
1.66	0.87	0.95
1.69	0.65	0.51
1.81	0.11	0.11
1.45	0.66	0.70
1.66	n.d.	0.97
1.15	n.d.	0.50
1.57	n.d.	1.19
1.61	n.d.	0.41
Sueros controles		
0.11	0.32	0.15
0.32	0.51	0.28
0.28	0.46	0.35
0.25	0.70	0.71
0.30	0.51	0.36
0.18	0.35	0.16

n.d. (no determinado)

DISCUSION

De un total de 270 sueros evaluados con el FT-ELISA (**Tabla 1**, pags. 12 y 13), se detectaron 66 sueros positivos procedentes de dos áreas endémicas para FT, México y Malasia. De estos sueros, 55 fueron hemocultivo positivo (grupos núm. 1 y 2); los sueros de 9 pacientes del grupo núm. 3, con hemocultivo negativo y Prueba de Widal positiva, también se consideraron verdaderos positivos ya que fueron diagnosticados como se reportó previamente (Pang y Puthucheary, 1983).

Con respecto a los otros dos sueros, el del grupo núm. 12 es un falso positivo confirmado, ya que procede de un individuo con fiebre paratifoidea, mientras que el otro (grupo núm. 4) podría ser un verdadero positivo, quizá con antibioterapia inadecuada, con lo cual el FT-ELISA podría estar detectando pacientes negativos por Prueba de Widal, lo cual se podría explicar debido a la poca sensibilidad de este método en áreas endémicas (Levine et al., 1978; Lambertucci et al., 1985). Aunque estos son hallazgos para investigación posterior (**Apéndice II**).

En relación a los 191 sueros controles, es importante resaltar que procedían de individuos de diferentes áreas geográficas, e incluyeron 43 donadores de sangre clínicamente sanos y 148 pacientes con una amplia gama de infecciones, tanto septicémicas como localizadas, causadas por bacterias Gram-positivas y -negativas, por virus, y por rickettsia, principalmente. De estos sueros controles, el único grupo sin un diagnóstico exacto, previo al FT-ELISA, fue el núm. 4, en el cual, los pacientes presentaban una sintomatología clínica que los hacía sospechosos de FT. Sin embargo, por el hecho de proceder de Malasia, una zona endémica (Chee et al., 1992), en la que se evaluaron con el FT-ELISA las otras enfermedades febriles de importancia regional, la probabilidad de que los individuos fueran falsos negativos se puede esperar que sea baja.

Desde el punto de vista práctico, se deben resaltar dos aspectos, por un lado el hecho de que el FT-ELISA haya sido eficiente para discriminar entre pacientes con FT y pacientes con otras infecciones en dos distintas áreas endémicas y, por otro lado, que el 100% de los sueros de pacientes diagnosticados por HC fueron positivos con el FT-ELISA, a una dilución única, de 1:3,125. Los elementos anteriores sugieren que el ensayo es un buen candidato para el diagnóstico rutinario de la FT. Como prueba alternativa

al HC, ofrece las ventajas de utilizar menos de 0.5 ml de sangre en una sola toma, contra 15-30 ml obtenidos de tres tomas para el HC, así como resultados en 4.5 horas contra 72 horas del HC. Así mismo, es importante señalar que el FT-ELISA fue sensible para diagnosticar pacientes con FT durante la primer semana de sintomatología (**Apéndice I**), así como hasta tres semanas después (**Apéndice II**).

Independientemente de los resultados mostrados aquí, como se menciona en los **Apéndices I, II y III** el FT-ELISA se debe continuar validando en diferentes áreas geográficas, en pacientes con salmonelosis (principalmente por *S. typhimurium*), con bacteremias diferentes a la FT, particularmente con fiebre paratifoidea, así mismo, con individuos en fase convaleciente de FT y con portadores sanos.

Otro elemento a considerar (**RESULTADOS** y **DISCUSION** del **Apéndice II**), es que con la utilización como antígeno, en el FT-ELISA, de pPME de *S. typhi* IMSS-1 (cepa mexicana de referencia) y pPME de *S. typhi* 100 (un aislado clínico de Malasia), no se observaron diferencias significativas entre los sueros de pacientes con FT. Con estos resultados, se sostiene el concepto de que *S. typhi* es una bacteria poco variable (Selander et al., 1990). Esto también es apoyado parcialmente por los resultados obtenidos en nuestro laboratorio por Puente et al., (1992), mediante la utilización de oligonucleótidos correspondientes a diferentes regiones de la secuencia de *ompC* de *S. typhi*, obtenidos al realizar hibridaciones con genomas de cepas de *S. typhi* procedentes de diversas regiones geográficas, así como de pacientes con distinta sintomatología clínica, habiéndose observado una alta conservación en el gene que codifica para dicha proteína.

Previamente, Selander et al., (1987), habían descrito una alta conservación en *S. typhi*, mediante estudios con isoenzimas. Con nuestros resultados apoyamos el concepto de que la conservación de la bacteria, no es únicamente a nivel de DNA total (Selander et al., 1990), de ciertas enzimas, y del DNA que codifica para una proteína expuesta en la membrana (Puente et al., 1992 y datos no publicados), sino además del perfil electroforético de PME totales.

Otro aspecto explorado mediante ELISA, fue la respuesta de anticuerpos de pacientes con FT, contra pPME de otras *Salmonella* diferentes a *S. typhi*, así como de *E. coli*. La pregunta que buscábamos responder era concerniente a las posibles reactividades

cruzadas de sueros de pacientes con infecciones con bacterias muy parecidas a *S. typhi*. Sin embargo, al carecer de los sueros, se exploró el camino indirecto mencionado, con resultados variables dependiendo de la cepa utilizada (**Tabla 2**, pag. 15), los cuales fueron desde 0% de reactividad cruzada (*E. coli, S. worthington, S. anatum*), hasta 70% (*S. typhimurium*).

Estos resultados no necesariamente significan que la observación sería igual en caso de contar con los sueros adecuados, sin embargo, en el **Apéndice I**, uno de los grupos de sueros control (IIa) fue el compuesto por turistas de EUA con diarrea (sin bacteremia) por *ETEC*, y no se detectó respuesta humoral cruzada. Así mismo, en la **Tabla I** (pag. 13, grupo núm. 13) se incluyeron cinco sueros procedentes de pacientes con bacteremia por *E. coli* y tampoco existió reacción, lo cual podría correlacionarse de manera congruente con lo observado en ELISA con las preparaciones de PME de *E. coli*. Otro ejemplo importante sería el mencionado en el mismo anexo y en la **Tabla 1** (pag. 12, grupo núm. 12), donde sueros procedentes de un brote por *Salmonella* grupo "B", tampoco mostraron reactividad cruzada en el FT-ELISA con pPME de *S. typhi*, que pertenece al grupo "D".

Al analizar las "reactividades cruzadas" detectadas en ELISA con pPME de distintas salmonelas, se debe considerar un elemento importante, como es el tipo de infección que causa cada cepa, y por ende, la interacción del patógeno con el sistema inmune del hospedero. Por ejemplo, las reactividades cruzadas mas importantes se observaron con pPME de *S. typhimurium* (73.6%), *S. paratyphi* B (57.8%), y *S. arizonae* (52.6%), sin embargo, la primera ocasiona principalmente una gastroenteritis aguda, y la última infecciones localizadas, mientras que *S. paratyphi* B, así como *S. paratyphi* A (21.0%) producen la fiebre paratifoidea (FPT), una fiebre entérica similar a la FT, razón por la cual, las validación del FT-ELISA con individuos con FPT es relevante. Como paradoja, en México y en las regiones endémicas para la FT, la FPT no es reportada como importante, quizá porque realmente no lo es, o porque el diagnóstico diferencial con la FT no es el adecuado. En este sentido, la pregunta de las reactividades cruzadas se ha contestado solo parcialmente.

Los resultados mostrados en la **Figura 3** del **Apéndice I**, así como en la **Figura 1**

de **RESULTADOS** (pag. 17), indicaron que la unión de los anticuerpos a los antígenos de captura (pPME o LPS) acoplados a la fase sólida, fue inhibida significativamente (mayor al 50%) por las pPME como antígeno libre, con respecto al LPS; sin embargo, la inhibición llevada a cabo por el LPS fue importante. Mas aún, en ensayos de inhibición en ELISA con antisueros de rata (**Figura 2**, pag. 18), la unión de los anticuerpos fue inhibida principalmente por pPME con respecto a LPSp.

Por otra parte, como se menciona en **RESULTADOS**, fue necesario utilizar una concentración 20 veces mas alta de LPS que la contenida en las pPME, como antígeno en el FT-ELISA, para que el valor de absorbancia de sueros de pacientes con FT fuera igual al detectado con el FT-ELISA, siendo la absorbancia de sueros de individuos negativos a FT por debajo de la línea de corte, aunque la desviación estándar fue mayor. De manera similar, Tsang et al., (1981), y Nardiello et al., (1984) emplearon LPS como antígeno en ELISA, y observaron una alta dispersión entre los sueros positivos evaluados, así como un cierto porcentaje de falsos positivos y de falsos negativos.

Considerando lo anterior, se ha observado un alto grado de sensibilidad con el uso LPS como antígeno en ELISA, sin embargo, la especificidad entre grupos de pacientes positivos y diferentes grupos de controles negativos a FT, ha sido menor que la obtenida por nosotros mediante la utilización de pPME.

Como se mencionó en la sección de **INTRODUCCION**, se han demostrado distintas funciones del LPS, concernientes principalmente a aspectos de resistencia al suero y lisis por complemento. Así mismo, se ha observado que bacterias con LPS incompleto son atenuadas en su virulencia. Sin embargo, un aspecto que, de alguna manera apoya nuestros resultados mediante inhibición-ELISA, es lo reportado por Sen y Nikaido (1991), quienes demostraron que cepas de *E. coli* con un fenotipo altamente rugoso, es decir, que tienen un LPS incompleto, son incapaces de ensamblar niveles normales de PME, especialmente porinas, en la membrana externa.

Debido a la variedad de funciones en patogenia adjudicadas al LPS, algunas de las cuales, también se les han adjudicado a las PME; y dados los hechos de que la trimerización de las porinas esté asociada a la interacción con el LPS y que la protección puede ser inducida por ambas clases de moléculas; así como por nuestros resultados, nos

quedamos con una pregunta abierta: si el LPS junto con las PME podrían estar formando epítopes complejos, o si tales complejos son necesarios para mantener inalterada a la proteína nativa o la estructura del LPS (**Apéndice I**, pag. 5).

Los resultados obtenidos en ELISA, cuando se utilizaron los péptidos OmpC-1 y OmpC-2 como antígeno en la placa, con respecto a los resultados obtenidos con las pPME (**Tabla 3**, pag. 20), mostraron que algunos sueros positivos en el FT-ELISA dieron un valor de absorbancia similar al de los sueros de pacientes positivos a FT, mientras que otros dieron un valor intermedio, y otros dieron un valor de absorbancia similar a los de los controles y, por otro lado, algunos sueros negativos, mostraron un valor de absorbancia cercano o mayor al punto de corte.

Una probable explicación a ésto, es que los sueros probados procedían de pacientes cuyo desarrollo de la enfermedad iba de una a cuatro semanas, y no se contaba con los datos exactos. Al respecto, se debe considerar que la duración de la exposición de un antígeno en un organismo implica que distintos mecanismos del sistema inmune del paciente se activan o desactivan conforme un antígeno permanece en el organismo. Por otra parte, el sistema retículoendotelial de cada individuo puede presentar variaciones, que a nivel de antígenos pequeños, como es el caso de péptidos, pueden encontrarse diferencias.

Así mismo, la conformación que adopta un péptido sintético, podría ser otro elemento que influyó en los resultados obtenidos. Por una parte, es bien sabido que únicamente 5% de la población de anticuerpos contra un antígeno en particular reconocen epítopes lineales, mientras que el 95% de los anticuerpos reconocen epítopes estructurales (Gam et al., 1992). Sin embargo, aunque la estructura primaria de los péptidos OmpC-1 y OmpC-2 fue deducida a partir de la secuencia de OmpC, la conformación adoptada por el péptido sintético, pudiera ser diferente de aquella encontrada en su entorno natural, en el cual, además, puede existir alguna clase de interacción con el LPS.

Nuestros resultados, de alguna manera, son similares a los reportados por Gam et al. (1992), quienes al utilizar sueros de pacientes con FT (hemocultivo positivos, de 1-4 semanas con FT) en ELISA, contra octapéptidos deducidos a partir de toda la secuencia

de OmpC de *S. typhi*, no encontraron que algún péptido en particular, fuera reconocido con mayor intensidad y de manera consistente por los anticuerpos de los pacientes.

Así mismo, en general se observó una respuesta similar contra ambos péptidos; es decir, aparentemente cuando el suero de un paciente reaccionó positivamente contra el péptido OmpC-1, también reaccionó contra el péptido OmpC-2.

Aun cuando los resultados obtenidos con los péptidos no han sido del todo homogéneos y, por ende, la interpretación de los mismos se torna compleja, fue importante el haber explorado en ELISA la respuesta inmune humoral contra antígenos expuestos pequeños. Al respecto, se puede concluir que este tipo de manipulación de moléculas puede enfrentarse a una diversidad de elementos, algunos de los cuales no son del todo controlables.

Si se piensa en la utilización de moléculas con fines diagnósticos o epidemiológicos en el area de las enfermedades infecciosas, es importante considerar a aquellas que puedan ser analizadas en un contexto con mínimas variables, y de ser posible controlables. En este sentido, si se compara el esquema de moléculas únicas y pequeñas para la captación de elementos generados por el sistema inmune de un paciente, por ejemplo los anticuerpos, con respecto a la captación de una molécula conservada del agente etiológico (péptidos, ADN, ARN), es muy importante contestar analíticamente esta clase de preguntas.

En conclusión, la aportación de este trabajo fue el diseño de un sistema nuevo para la detección rápida y precisa de la FT, que se debe continuar validando en diferentes laboratorios del mundo. Conceptualmente, podemos decir que la respuesta inmune humoral sérica específica en contra de las proteínas de la membrana externa, depende de conformaciones e interacciones que todavía deben ser elucidadas.

Apéndice I

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Early Diagnosis of Typhoid Fever by an Enzyme Immunoassay Using *Salmonella typhi* Outer Membrane Protein Preparations

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An enzyme immunoassay (EIA) for detection of serum antibodies in patients with typhoid fever was developed using *Salmonella typhi* outer membrane protein (OMP) preparations as antigen. Acute phase (first week) sera from adult typhoid fever patients were tested as well as sera from the following control groups: adult travellers with diarrhea caused by enterotoxigenic *Escherichia coli*, children infected with *Campylobacter jejuni*, healthy Mexican adult blood donors, and adults with septicemia caused by other organisms. At a 1:3,125 serum dilution, the mean absorbance values were 1.41 in the typhoid fever patients, and 0.57, 0.55, 0.51 and 0.52 in the respective control groups. Inhibition EIA studies using OMP preparations or lipopolysaccharide (LPS) as free antigen indicated that proteins can play an important role in the detection of antibodies in early typhoid fever. This EIA may be useful for the diagnosis of typhoid fever since results were obtained within about five hours and in an endemic area antibodies against *Salmonella typhi* OMP preparations appear early in the course of the disease.

Typhoid fever in man is the clinical manifestation of a systemic infection with *Salmonella typhi*. At present this disease is endemic in developing countries, where sanitary conditions are suitable for its propagation. It been estimated that worldwide, excluding China, 12.5 million persons suffer from typhoid fever annually. The worldwide case-fatality rate is around 1 % (1).

At present, several tests can be recommended for the diagnosis of typhoid fever. The single most effective method of diagnosis is bone marrow culture, with an efficiency of around 90 %. Bone marrow puncture is, however, an invasive and impractical procedure. Blood cultures are more commonly used and easy to perform, but to attain a sensitivity of over 80 % (in the absence of antimicrobial therapy) a set of up to three blood samples has to be cultured during the first week of illness. Furthermore, with this method, isolation and identification of *Salmonella typhi* takes at least 48 h, and blood cultures may not be sensitive enough due to the low concentration of *Salmonella typhi* in blood (20 cells/10 ml at most), especially when the patient has taken antibiotics (1–3).

One of the most widely used serodiagnostic methods is the Widal test, but it has low specificity in endemic areas due to a high background of lipopolysaccharide (LPS) side-chain and H flagellar antibody titers in healthy adults (4). Even so, it has been successfully used in endemic areas by carefully defining the agglutinin levels in normal individuals (5). Identification of *Salmonella typhi* in cultures of the mononuclear cell-platelet fraction of blood has been reported (6). An EIA technique using monoclonal antibodies for detecting *Salmonella typhi* flagellin in patient sera shows high sensitivity and specificity (7), and represents a considerable improvement over EIAs based on the detection of total protein or Vi capsular antigen (8, 9).

Antibodies to various *Salmonella typhi* antigens have been detected by EIA in typhoid fever patients. The immune response to crude protein or LPS has been varied, making it difficult to establish a cut-off value between true positive and negative samples (10–13). Detection of antibodies to Vi antigen in typhoid fever patients is not very sensitive, but has proven to be more sensitive in the detection of chronic carriers (14–17).

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The use of cell envelope fractions, or outer membrane proteins (OMPs), appears to increase the sensitivity of the EIA (18, 19). The immune response in typhoid fever patients to OMPs has been shown on immunoblot (20, 21).

Detection of *Salmonella typhi* in blood samples of typhoid fever patients by DNA hybridization has been done with the gene coding for the *Salmonella typhi* capsular Vi antigen, and work is in progress in order to improve the sensitivity and specificity of this technique (22). The potential use of the *Salmonella typhi fliC* flagellin gene in molecular epidemiology has been illustrated in the polymerase chain reaction (23).

We report here on an EIA for detection of antibodies against *Salmonella typhi* OMP in serum of typhoid fever patients in the first week of disease, which could be used routinely for rapid and specific diagnosis.

Materials and Methods

Sera. Human sera were collected either from patients or from blood donors at the Instituto Nacional de la Nutrición "Salvador Zubirán", México City. Blood was drawn during the period of clinical signs of infection as indicated for each of the following groups: Group I consisted of typhoid fever patients who were Mexican adults (18 to 45 years old) with a positive *Salmonella typhi* blood culture; sera were taken in the first week of clinical signs. The control groups consisted of: group IIa: adult USA travellers with acute diarrhea for 3–4 days and stool cultures positive for enterotoxigenic *Escherichia coli* (ETEC); group IIb: Mexican children (1–2 years old) with stool cultures positive for *Campylobacter jejuni* clinical signs for 3–4 days; group IIc: healthy Mexican adult blood donors (age- and sex-matched with group I); group IId: Mexican adults with septicemia caused by other organisms; sera were taken in the first week of clinical signs.

Strains. Salmonella typhi IMSS-1, a clinical isolate from a patient with typhoid fever (24), was kindly provided by Dr. J. Kumate of the Instituto Mexicano del Seguro Social, Mexico City. The *Salmonella typhi* Ty 2 strain used corresponds to reference strain NCTC 8385 from the American Type Culture Collection. All other *Salmonella typhi* strains were isolated and serotyped at the Department of Infectious Diseases at the Instituto Nacional de la Nutriefon "Salvador Zubirán", México City.

Bacterial Culture. Organisms were grown at 37 °C up to the late logarithmic phase in medium A, containing per liter 7 g Nutrient Broth (Difco, USA), 1 g Yeast Extract (Difco), 2 ml glycerol, 3.7 g K_2HPO_4, 1.3 g KH_2HPO_4. Culture conditions were varied by the addition of either 2.5 mg/ml of human or chicken egg white transferrin (conalbumin), or 100–150 μM 2,2,dipyridyl (Sigma, USA) to chelate free iron.

Outer Membrane Protein Preparations. OMP preparations were obtained as described previously (25). Briefly, cells were washed and resuspended in 10 mM Na_2HPO_4 (pH 7.2) and disrupted by sonication over ice (seven 30 sec pulses at 30 sec intervals). The membrane fraction was recovered by centrifugation at 40,000 rpm for 30 min in the 55.2 Ti rotor (Beckman, USA) and then resuspended in 2 % Triton X-100, 10 mM Na_2HPO_4 (pH 7.2). After incubation at 37 °C for 15 min, the Triton X-100-insoluble fraction was recovered by centrifugation at 40,000 rpm for 30 min. This pellet was finally washed in 10 mM Na_2HPO_4 (pH 7.2) and resuspended in PBS of pH 7.4, containing per liter 16 g NaCl, 5.8 g Na_2HPO_4, 6.4 g KH_2PO_4, 0.4 g KCl, and B-1 solution (1 % SDS, 1 % 2-mercaptoethanol).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The OMP profile was analyzed by SDS-PAGE. OMP preparations were boiled for 5 min, dissolved in sample buffer (1 % SDS, 1 % 2-mercaptoethanol, 10 M urea, 0.005 % bromophenol blue; pH 6.8), and then applied (around 10 μg of OMP per lane) to an 8–15 % or 10–20 % continuous gradient polyacrylamide (30:0.8 acrylamide to bisacrylamide) 0.1 % SDS slab (10 x 15 cm x 3 mm) gel, and subjected to electrophoresis for 5 h at 20 mA in the discontinuous buffer system (26).

Protein Measurement. For OMP preparations, protein was measured by a standard procedure (27). Measurement of the protein content in the commercial LPS (Sigma L2387) used was done as described previously (28).

Lipopolysaccharide Measurement. LPS quantification in the OMP preparations, using commercial LPS as the standard, was done by measuring the 2-keto-3-deoxyoctonate (KDO) content as described previously (29).

Typhoid Fever Enzyme Immunoassay (TF-EIA). General procedures were as described previously (30). Briefly, EIA microtiter plates (Cat. No. 373660, Beckman Scientific Instruments, USA; or Cat. No. 4-39454, Nunc, Denmark) were coated with either OMP preparations (5 μg of protein per ml) or LPS (35 μg/ml) in PBS (100 μl per well) and incubated at room temperature overnight. One hundred microliters of diluted human sera were used per well; the titer selected for OMP preparations was 1:3,125, whereas the titer for LPS was 1:625. Each specimen was assayed in duplicate and incubated at room temperature for 90 min. The anti-human immunoglobulin-horseradish peroxidase-conjugated immunoglobulins which were protein A chromatography-isolated rabbit anti-IgA, -IgG, -IgM (Cat. No. P212, Dako, USA; or Cat. No. AXL335p, Axell Accurate Chemical & Scientific, USA), and ortho-phenylenediamine-H_2O_2 enzyme substrate were used as described previously (31). The substrate-enzyme reaction step was performed for 20 min. The cut-off absorbance value was the mid-point between the mean absorbance of the positive typhoid fever sera and the mean absorbance of the control sera.

The proportion of immunoglobulin isotypes for typhoid fever was determined as described above. The working dilutions of human sera for IgG were also 1:3,125; those for IgM and IgA were changed to 1:625; anti-IgM and

anti-IgA-conjugates were diluted 1:500 and 1:250, respectively. Anti-human immunoglobulins were horseradish peroxidase-conjugates (rabbit antibody anti-*gamma*, -*mu* and -*alpha* chains; Cat. No. P214, P215 and P216, Dako).

Inhibition Enzyme Immunoassay. The capability of either OMP preparations or LPS to inhibit the binding of anti-OMP antibodies to solid-phase bound OMP preparations or LPS was assayed by an inhibition EIA method. Human sera were diluted 1:625 or 1:125 to test inhibition of binding to OMP preparations or to LPS, respectively. These serum dilutions were concentrated five-fold with respect to the EIA titers. Free antigen (OMP preparation or LPS) was present in two-fold serial dilutions, ranging from 25 to 0.39 µg/ml. The serum-antigen mixtures were incubated at room temperature for 90 min, and the plates developed as described above for the TF-EIA. The antigen concentration was determined that inhibited the binding of the respective human antibodies to solid phase-bound OMP preparations or LPS by 50 %.

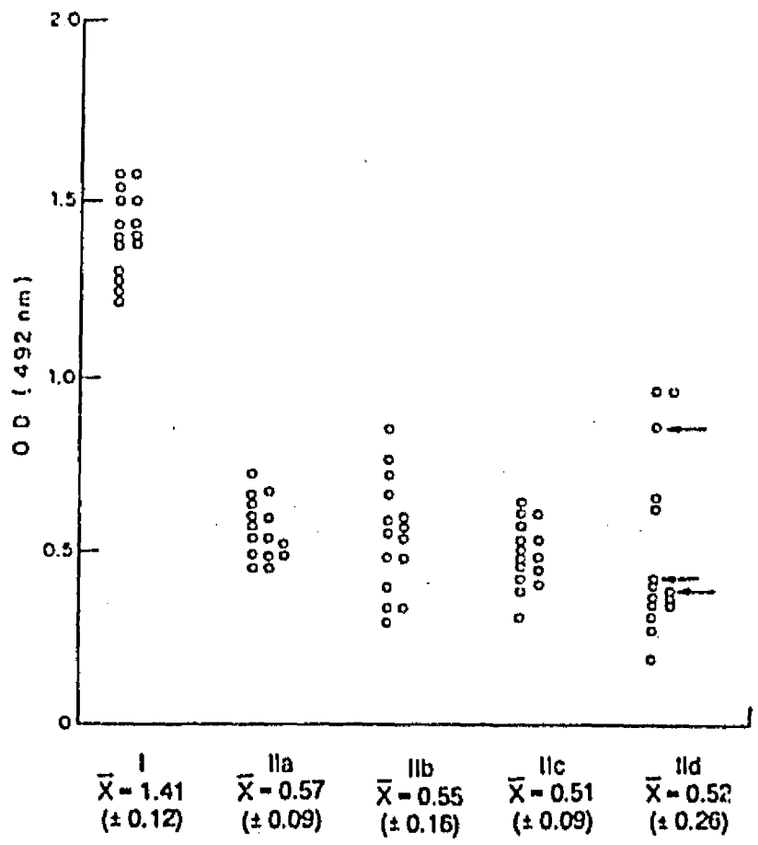

Figure 2: TF-EIA. Absorbance values for sera from different subject groups at a single (1:3,125) serum dilution. I: First week adult typhoid fever patients with a positive blood culture. Control groups: IIa: adult USA travellers infected with enterotoxigenic *Escherichia coli*; IIb: children infected with *Campylobacter jejuni*; IIc: healthy adult blood donors; IId: adults with septicemia caused by other organisms, including *Salmonella enteritidis*, *Salmonella paratyphi* B, *Salmonella* spp., *Proteus* spp., *Candida albicans*, *Shigella flexnerii* and *Escherichia coli*, the first three being indicated by arrows in descending order.

Results

Outer Membrane Protein Preparations. On SDS-PAGE, three major proteins were observed in OMP preparations from *Salmonella typhi* IMSS-1 grown in nutrient broth (Figure 1, lanes a and b). The apparent molecular weights were 38.5 kDa, corresponding to OmpC (32); 37.5 kDa, corresponding to OmpF on the basis of its repression in high osmolarity (33, 34); and 34.5 kDa, identified as OmpA on the basis that it is constitutive and shows heat-modifiable electrophoretic mobility, i.e. it migrates with an apparent molecular weight of 31.5 kDa when it is not heated (not shown). In addition, several minor polypeptides were observed with apparent molecular weights ranging from 29 to 103 kDa. OmpC and OmpF were resolved in 10 to 20 % gradient polyacrylamide gels (Figure 1, lane a), whereas in 8 to 15 % gradient gels, utilized for resolving high molecular weight bands, they migrated as a thick band (Figure 1, lanes b and c).

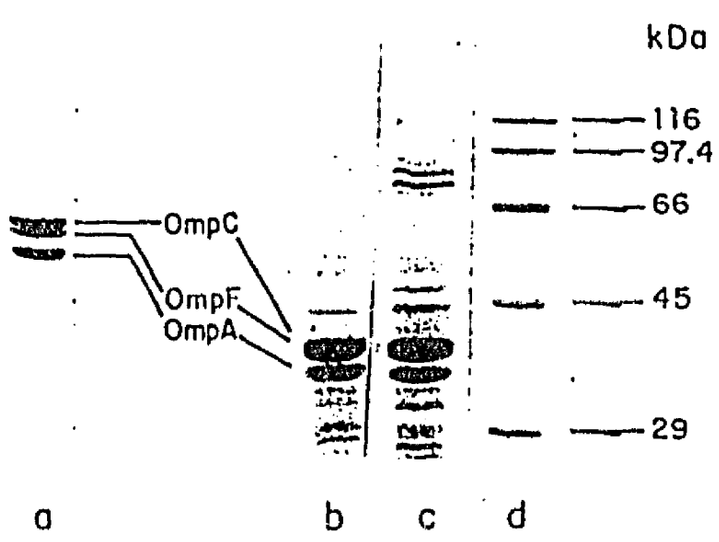

Figure 1: SDS-PAGE profiles of outer membrane protein (OMP) preparations from *Salmonella typhi* grown in a nutrient medium (medium A) without (lanes a, b) or with (lane c) 2.5 mg/ml human transferrin, a free-iron chelator. Protein molecular weight markers are included (lane d). Lane a corresponds to a 10–20 % polyacrylamide gradient gel; and lanes b-d to an 8–15 % gel.

No apparent differences were observed between the above OMP electrophoretic profile and those for *Salmonella typhi* Ty 2 or for clinical isolates from typhoid fever patients, either with hepatitis or with hemorrhagic enteritis, as well as from a healthy carrier (not shown). The OMP profiles obtained for organisms cells grown in the presence of any of the iron chelators showed over-expression of various high molecular weight polypeptides, mainly seven of approximately 53, 72, 77, 82, 92, 97.5 and 103 kDa (Figure 1, lane b), aside from the three above-mentioned major OMPs.

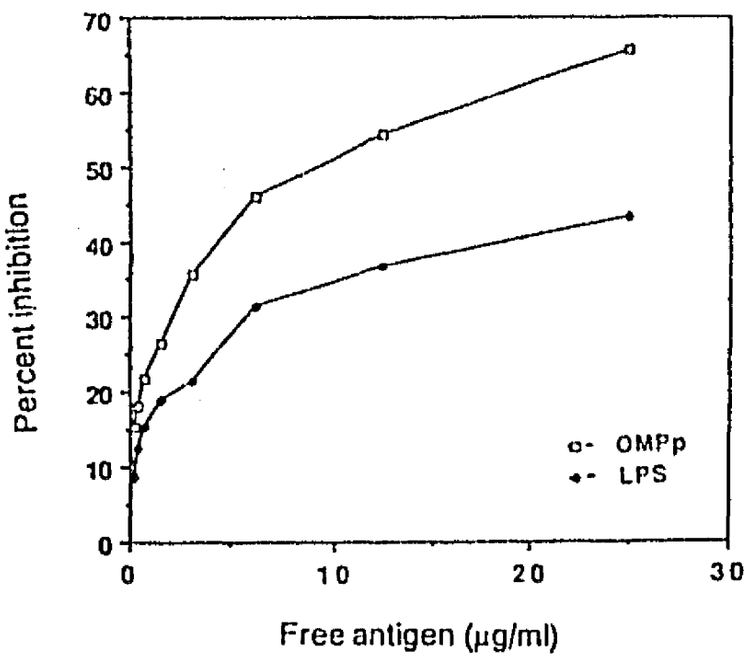

Figure 3: Inhibition EIA. Inhibition of the binding of antibodies from typhoid fever patient sera to solid phase-bound *Salmonella typhi* OMP preparations at different concentrations (0.19–25 µg/ml) of free antigen. Typhoid fever sera were diluted 1:625 or 1:125 when used with an OMP preparation or LPS as free antigen, respectively.

The OMP preparations had a 30 % w/w LPS content, determined as described in the Materials and Methods. In addition, we determined that the commercial LPS contained less than 10 ng of protein per µg of LPS.

Typhoid Fever Enzyme Immunoassay. The absorbance values obtained in the TF-ELISA, using OMP preparations as antigen, are plotted in Figure 2. We observed a statistically significant difference (*U* of Mann-Whitney) (35) with a p value of < 0.01 between group I typhoid fever patients and control groups IIa, IIb and IIc; the difference between group I patients and control group IId patients with septicemia had a p value of < 0.05. The absorbance values in patients with septicemia caused by *Salmonella enteritidis*, *Salmonella paratyphi* B and *Salmonella* spp. were 0.86, 0.44 and 0.4, respectively, and are indicated with arrows in Figure 2. The overall proportion of immunoglobulin isotypes was found to be 60 % IgG, 20 % IgM and 12 % IgA in sera of typhoid fever patients.

OMP preparations obtained from cultures grown in the presence of an iron chelator rendered absorbance values that were 5 % higher than in cultures without iron chelator.

Inhibition Enzyme Immunoassay. To evaluate the role of LPS in the antibody response detected by the TF-EIA, we designed an inhibition EIA. Fifty percent or more inhibition of the human antibody response to solid phase-bound OMP preparations (5 µg/ml of protein) was observed with approximately 9 µg/ml or even less of soluble OMP preparations, whereas soluble LPS did not inhibit by 50 % even with 25 µg/ml (Figure 3).

Discussion

The TF-EIA described here differs from other techniques with respect to the antigen used (8, 10, 14, 18, 36). This assay could be useful for routine early diagnosis of typhoid fever in endemic areas since it showed high specificity (100 %) and high sensitivity (100 %) compared with isolation of *Salmonella typhi* from blood cultures. A clear advantage of the TF-EIA is the reduction in the performance time (4.5 hours), in contrast with the blood culture technique which requires at least 48 hours. To date, only a group working with *Bacteroides nodosus* has successfully used an OMP-EIA as a diagnostic method (37).

In the present study the arithmetic mean of the TF-EIA absorbance values for the typhoid fever patient group were 2.5 to 2.8 times higher than the mean values for control groups. The absorbance values with the highest dispersion were observed with sera from non-typhoid septicemia patients, although the difference in the absorbance values compared with the typhoid fever group was sufficient to discriminate between them. Blood culture confirmation was used as the reference method for positive typhoid fever sera since the Widal test appears to lack positive predictive value in endemic areas (4, 38).

Calderón et al. (19) described an EIA in which IgG and IgM against *Salmonella typhi* porins were detected, and found that absorbance values with porins from typhoid fever patients were significantly different from those obtained in sera from clinically healthy individuals in an area endemic for typhoid fever. Nevertheless, they did not evaluate non-typhoid septicemia patients. More recently, Appassakij et al. (8) designed an EIA to detect protein antigen in serum; their results varied widely, and a certain degree of cross-reactivity with paratyphoid fever patients and healthy controls was seen.

With regard to cross-reactivity in the EIA in this study, absorbance values in sera from three individuals with septicemia caused by other salmonellae were lower than those obtained in sera of typhoid fever patients (Figure 2, arrows). In

addition, we used our TF-EIA to test sera from six individuals suspected of having either salmonellosis or typhoid fever and residing in a rural Mexican community where there had been an outbreak of diarrhea. The results were negative. Subsequently, *Salmonella* group B was isolated from the stools of three of these patients; the rest of them were negative on stool culture (data not shown). Furthermore, the TF-EIA described here has been used with success in typhoid fever patients in Malaysia (unpublished data). In this study, we also assessed the cross-reactivity in sera from 16 patients with blood culture-confirmed typhoid fever using other OMP preparations from gram-negative bacteria. No cross-reactivity was observed with *Escherichia coli*, *Salmonella aberdeen* or *Salmonella worthington* OMP preparations. Variable cross-reactivity was seen with OMP preparations from *Salmonella paratyphi* A, *Salmonella anatum*, *Salmonella paratyphi* B and *Salmonella typhimurium* (12.5 %, 25 %, 50 % and 56 %, respectively).

The proportion of immunoglobulin isotypes detected in patients with *Salmonella typhi* infection is similar to that reported previously by other authors (18, 19, 39). Specifically, Calderón et al. (19) have shown that *Salmonella typhi* porins are recognized mainly by IgG, and to a lesser extent by IgM, when using sera from typhoid fever patients.

In this study, the minimal concentration of OMP preparation for 50 % inhibition of antibody binding to OMP or LPS was 2.5 to 3.3 times lower than when LPS (less than 10 ng/µg protein content) was used as an inhibitor. These results support the concept that proteins can play an important role in the detection of the humoral immune response, as measured by the TF-EIA described here. Nevertheless, it remains an open question whether LPS together with OMPs might be forming complex epitopes, or whether such complexes are necessary to maintain unaltered native protein or LPS structures. Sen and Nikaido (40) showed that cells producing defective LPS molecules cannot assemble normal levels of proteins, especially porins, in the outer membrane.

In protection studies, Isibasi et al. (24), using a mouse model, found that *Salmonella typhi* porins (containing 4 % LPS by weight) were highly immunogenic and protective. Nevertheless, they observed a protective specific immune response mediated by LPS, although 40 % lower than that obtained with OMPs. Elsewhere, Udhayakumar and Muthukkaruppan (41), using *Salmonella typhimurium* OMPs and LPS in a mouse model, reported that the protective immunity induced by the protein fraction was greatest. However, they observed that the presence of LPS was necessary to generate the protective activity by one of the membrane proteins (a porin).

The electrophoretic profiles of OMP preparations obtained from cells grown in the presence of an iron chelator showed over-expression of mainly seven high molecular weight polypeptides, aside of the three major OMPs (Figure 1, lane b). In a similar study, Fernández-Beros et al. (20) described three polypeptides of 69, 78 and 83 kDa that are over-expressed in the presence of an iron chelator. Four more protein bands may have been observed in this study due to the fact that gradient 8–15 % polyacrylamide gels were used instead of non-gradient gels.

Further efforts in identifying the major antigens of the OMP preparations are needed. More extensive epidemiological studies with sera from typhoid fever patients at different stages of the disease, including convalescence, should be of value for assessing the utility of the TF-EIA. In addition, studies with sera from chronic *Salmonella typhi* carriers, typhoid fever patients from other geographical areas and individuals with a more diverse panel of infections, especially paratyphoid fever, as controls are needed to further test the validity of this TF-EIA.

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Apéndice II

FACSIMILE TRANSMISSION

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TEXT:

Dear Edmundo,

Many thanks for your courier-delivered note dated April 15.

1. I have passed the document to Mr Teoh Beng Kuan at our examinations section.

2. With regards to our joint manuscript : I have submitted the revised version to the Editor in Thailand (within a week of receiving the materials from you). I have not heard from them regarding acceptance of the revised version. However, from past experience with this Journal, the next communication is in the form of galley proofs. I will try to find out what is happening. I am fairly sure that our revised manuscript would have satisfied the referees.

With best wishes,

T. Pang

Detection of Antibodies against *Salmonella typhi* Outer Membrane Protein (OMP) preparations in typhoid fever patients

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Running title: Antibodies to *S. typhi* OMPs

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Summary

An indirect ELISA was developed to detect antibodies against outer membrane proteins (OMP) preparations from *Salmonella typhi*. Sera from patients with a definitive diagnosis of typhoid fever (TF) gave a mean absorbance reading, at 414 nm, of 1.52 $\pm$ 0.23 as compared to 0.3 $\pm$ 0.11 for sera from healthy individuals. This gave a positive to negative ratio of absorbance readings of approximately 5.1. Suspected TF patients (no isolation of *S. typhi*) with positive and negative Widal titres had mean absorbance reading of 1.28 $\pm$ 0.46 and 0.25 $\pm$ 0.19, respectively. Sera from patients with leptospirosis, rickettsial typhus, dengue fever, and other infections gave mean absorbances of 0.20 $\pm$ 0.08, 0.24 $\pm$ 0.08, 0.27 $\pm$ 0.08, and 0.31 $\pm$ 0.16, respectively. The sensitivity, specificity, positive and negative predictive values were 100%, 94%, 80% and 100%, respectively. The antibody response detected in the definitive TF cases was predominantly IgG in nature and no cross-reactivity was seen with OMP preparations extracted from *E. coli*. Variable reactivity was noted with OMP preparations obtained from other *Salmonella* spp. Three major OMPs are present in the antigen preparation and strong binding of positive sera was detected to all three bands.

Introduction

The serological diagnosis of typhoid fever still relies largely upon the Widal test, which detects the presence of antibodies to the O (lipopolysaccharide) and H (flagellin) antigens derived from *Salmonella typhi*. This test, however, is difficult to interpret in endemic areas, due to the high background titres among the population[1,2]. As a result, newer methods have been developed which are based on detection of antibodies to the protein antigens of *S. typhi*[3]. For example, it has been suggested that outer membrane proteins (OMPs), which are abundant and exposed on the surface of the cell, might be of diagnostic value. OMPs have been shown to induce protective immunity to *S. typhi* and *S. typhimurium* in experimental models[4,5,6], and there appears to be a significant increase in antibody titres to OMPs in acute-phase and convalescent-phase sera as compared with normal sera, and these antibody levels appear to be higher than antibody titres to O and H antigens[7]. An indirect ELISA has been previously developed for detection of serum antibodies against OMPs, with results that allowed discrimination between groups of TF-positive and TF-negative subjects[8,9]. Further evaluation of this test, especially in endemic areas, is both necessary and desirable in the quest for better serological tools in the diagnosis of TF. We report here the results of a study to evaluate the indirect ELISA in the detection of antibodies against *S. typhi* OMP in TF patients from two endemic areas, Mexico and Malaysia.

Materials and Methods

Strains

Salmonella strains:

1) *S. typhi* 100 is a Malaysian isolate (this study).

2) *S. typhi* IMSS-1 is a Mexican reference strain[4,8,10].

3) *S. typhimurium* LT2 is the original strain of Herzberg obtained from the collection of Dr. Bruce Stocker (SL937), Stanford University, U.S.A.

4) *S. worthington* serotype G2 (1, 13, 23, z) is a Mexican clinical isolate from the Instituto Nacional de la Nutrición "Salvador Zubirán", Mexico City.

5) *S. paratyphi* serotype A (1, 2, 12)

6) *S. paratyphi* serotype B (1, 4, [15], 12)

7) *S. anatum* serotype E1 (3, 10e, h1, 6)

8) *S. aberdeen* serotype F (11, i, 1, 2)

Numbers 5-8 correspond to reference strains from Bigaux Diagnostica, Mexico City.

Escherichia coli HB101, has been described previously[11].

Sera

Several groups of human sera were used in the study. Group 1 consisted of 41 sera collected from patients with a definitive diagnosis of TF as indicated by a positive Widal test and isolation of *S. typhi* from the blood and/or stool. Sera were collected at various times ranging from 1 week to 1 month after onset of disease. This group consisted of 38 Malaysian samples, and three control serum samples from blood culture-confirmed

Mexican TF patients. Group 2 consisted of eleven sera from suspected cases of TF with positive Widal titres, but negative blood cultures. Group 3 comprised 48 sera from suspected TF cases, but with negative Widal titres, and negative blood cultures. The method of doing the Widal test, as well as the titres considered to be significant, have been reported previously[2]. Group 4 consisted of 28 serum samples from healthy blood donors. Group 5 is made up of sera collected from patients with other febrile illnesses common in the region, including dengue fever (9 samples), leptospirosis (17 samples) and rickettsial typhus (19 samples). Diagnosis of these illnesses were made on the basis of standard serological tests or isolation of the causative organism. Group 6 consisted of 30 Mexican patients with other infections, where the organism was identified by isolation, including bacteremia by: *S. enteritidis*, *S. paratyphi* B, *Salmonella* spp., *Proteus* spp., *Shigella flexnerii*, *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Staphylococcus* coagulase (+), *Staphylococcus* coagulase (-), *Enterococcus* group D, *Streptococcus* group B, *Acinetobacter coalcoaceticus*; septicemia by *Candida albicans*; and six serum samples from patients involved in a diarrheal outbreak by *Salmonella* group B.

Preparation of OMPs

Seven hour cultures of bacterial cells in nutrient broth were centrifuged at 1,400g at 4°C for 10 min. The bacterial cell pellet was then resuspended in phosphate-buffered saline (275 mMNaCl, 40mM Na_2HPO_4, 37mM KH_2PO_4, 5.4mM KCl, pH 7.4; PBS) and sonicated at a setting of 20kHz or 20,000 cyles/sec (Vibra Cell sonicator, Sonic & Material Co., Danbury, Connecticut, U.S.A.). Sonicated cells were centrifuged at 1,400g at 4°C for 10 min, and the obtained supernatant was centrifuged at 100,000g

at 4°C for 30 min and the pellet resuspended in a small volume of PBS containing 2% Triton X-100 and incubated at 37°C for 20 min. The centrifugation step was repeated and the pellet resuspended in PBS and stored at -20°C until use. Protein concentration of the OMP preparation was measured by the Bradford method[12], and lipopolysaccharide (LPS) content by 2-keto-3-deoxyoctonate (KDO) determination[13].

ELISA testing

Microtitre plates (96-well, flat-bottom, Nunc Plate, Intermed, Denmark) were coated overnight at room temperature with 150 µl OMP preparation (equivalent to 2.5 µg/ml protein). Plates were washed 3 times the next day with PBS containing 0.1% Tween-20 (PBS-Tween). Blocking was then carried out by addition of 150 µl PBS containing 2% BSA, 0.1% Tween-20, and incubating for one hr at room temperature followed by three washes with PBS-0.1% Tween-20. Primary antibody (human sera) was then added (100 µl/well of a 1 in 3,125 dilution) and the plates incubated for 1 hr at 37°C. After 4 washes with PBS-0.1% Tween-20, 100 µl of secondary antibody (1 in 1,000 dilution, goat anti-whole human serum, KPL Co., Gaithersburg, MD, U.S.A.) were added to each well and the plates incubated for one hr at 37°C. Plates were then washed 5 times with PBS-Tween before addition of 100 µl of substrate (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, ABTS, Sigma Chemical Co., St. Louis, MO, U.S.A., at 0.5 mg/ml). Colour was allowed to develop for 45 min at room temperature and absorbance at 414 nm measured in a Titertek Multiskan MCC-340 ELISA reader (Flow Laboratories, Australia). Where indicated, affinity purified, horseradish peroxidase-conjugated rabbit anti-human IgA, IgM or IgA (Dakopatts A/S, Glostrum, Denmark) were also used as secondary antibody in the ELISA.

The cutoff absorbance value, between TF patients and controls, was the mid-point between the mean absorbance of the positive TF sera (Group 1)) and the mean absorbance of the healthy blood donors (Group 4). The gold standard was defined as febrile patients with a clinical setting corresponding to TF and both a positive blood and/or stool culture and Widal test (Group 1).

Polyacrylamide gel electrophoresis (PAGE) analysis & immunoblotting

SDS-PAGE in 12% polyacrylamide gels, containing 0.01% sodium dodecyl sulphate (SDS), were used to analyze the OMP preparations. Ten µg of protein contained in an OMP preparation (in PBS, 1% SDS and 1% 2-mercaptoethanol) were boiled for 5 min prior to loading on the gel. Electrophoresis was in 0.025M Tris (pH 8.3), 0.192M glycine, 0.1% SDS for 3 hr at 170-250 V. Gels were then stained with 0.1% Coomassie blue followed by destaining with 40% methanol-7% acetic acid. Molecular weight markers ranging from 29 to 205 kDa were used (Sigma Chemical Co., St. Louis, Mo., U.S.A.). Transfer of separated proteins to nitrocellulose membranes was carried out by a semi-dry method in a SemiPhor TE7O system (Hoefer Scientific Instruments, San Francisco, CA., U.S.A.) for 3 hr at 134mA. After transfer, membranes (10 x 14 cm) were blocked with TNT (10mM Tris, 150mM NaCl, 0.05% Tween-20) containing 3% BSA (TNT-BSA) for 1.5 hr at room temperature. Primary antibody (pooled sera from Group 1 patients) was then added at a dilution of 1 in 400, and incubated overnight at room temperature with gentle shaking. Membranes were washed 3 times with TNT-BSA followed by addition of biotinylated goat anti-whole human serum (1 in 1,000 dilution, Clontech Laboratories Inc., Palo Alto, CA., U.S.A.) and incubated for 2.5 hr at room temperature. After 3 washes

with TNT-BSA, streptavidin-alkaline phosphatase conjugate (1 in 1,000 dilution, ImmunoSelect, Life Technologies Inc., Gaithersburg, MD, U.S.A.) was added and incubation carried out for 10-15 min at room temperature. Membranes were then washed twice with 0.1M Tris (p H7.5), 0.15M NaCl for 10 min and twice more with 0.1M Tris (pH 9.5), 0.1M NaCl, 50mM $MgCl_2$ for 10 min. Substrate was then added (NBT-BCIP, nitroblue tetrazolium Cl-5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, Immunoselect, Life Technologies Inc., Gaithersburg, MD, U.S.A.) at a concentration of 132 µl of NBT, 102 µl of BCIP in 40 ml of 0.1M Tris (pH 9.5), 0.1M NaCl, 50mM $MgCl_2$ and colour development allowed to proceed for 10-30 min. The reaction was stopped by addition of 20mM Tris (pH 7.5), 0.5M EDTA.

Inhibition ELISA.

The capability of either OMP preparations or LPS (Sigma Chemical Co., St. Louis, Mo., U.S.A.), to inhibit the binding of anti-OMP antibodies to solid-phase bound OMP preparations (5 µg/ml), was assayed by an inhibition ELISA method. Human sera were diluted 1:625 or 1:125, when testing binding inhibition to OMP preparations or to LPS, respectively. These serum dilutions were five-fold concentrated with respect to the ELISA titres. Free antigen (OMP preparations or LPS), was present in two-fold serial dilutions, ranging from 25 to 0.39 µg/ml. The serum-antigen mixtures were incubated at room temperature for 90 min, washed, and 100 µl of diluted 1:1000 anti-human immunoglobulin-horseradish peroxidase-conjugted immunoglobulins (Cat. P212 from DAKO Corporation, USA) were added per well, incubated for 90 min at room temperature, and the plates were developed with 100 µl per well of peroxidase substrate solution: 38mM $HOC(COOH)(CH_2COOH)_2$, 83mM Na_2HPO_4, pH 5.5; 1 mg/ml *ortho*-phenylenediamine (Sigma

Chemical Co), 0.4 µl/ml 30% H_2O_2. The antigen concentration that inhibited by 50% the binding of the respective human antibodies, to solid phase-bound OMP preparations, was determined.

Results

SDS-PAGE analysis of the OMP preparation indicated the presence of three major OMPs, OmpC, OmpF and OmpA, with apparent molecular weights of 38.5, 37 and 34 kDa, respectively, and several minor polypeptides ranging from 18 to 103 kDa (Fig. 1).

ELISA testing (Fig. 2 and Table I) of various sera with OMP preparation-coated plates showed significantly stronger binding of sera from confirmed cases of TF (Group 1) as compared to sera from healthy blood donors (Group 4), with mean absorbance (at 414 nm) of 1.52 $\pm$ 0.23 and 0.30 $\pm$ 0.11, respectively, and giving a positive:negative absorbance ratio of 5.1. No differences were observed in the binding of sera from Mexican or Malaysian patients. Suspected TF patients with positive (Group 2) and negative (Group 3) Widal titres, but negative blood cultures, had a mean absorbance of 1.28 $\pm$ 0.46 and 0.25 $\pm$ 0.19, respectively. Sera from patients with other fevers common in the region (group 5), namely leptospirosis, rickettsial typhus and dengue fever, showed no significant binding to OMP preparations. Sera from Mexican patients with other infections (Group 6), had a mean absorbance of 0.31 $\pm$ 0.16.

The calculated sensitivity in the ELISA was 100%, the specificity 94%, and the positive and negative predictive values were 80% and 100%, respectively. No significant differences were found in the ELISA, nor in the electrophoretic OMP profile, when OMP preparations were from either the Mexican reference strain or the Malaysian isolate of *S. typhi*.

Testing of Group 1 sera with affinity-purified, rabbit anti-human IgG, IgM, and IgA as secondary antibody showed that the proportion of the immunoglobulin isotypes in the response was approximately 60%, 20%, and 12%, respectively (data not shown).

Testing of sixteen Group 1 sera, in ELISA, with OMP preparations from various bacteria, showed no cross reactivity with an OMP preparation from *E. coli*, although variable reactivity with OMP preparations from other *Salmonella* spp. was observed (Table II).

The inhibition-ELISA was designed to assess the participation of LPS in the antibody response detected. A greater than 50% inhibition of the human antibody response, to solid phase-bound OMP preparation, was observed with 9 μg/ml at most of soluble OMP preparations; in contrast, soluble LPS did not inhibit 50% even with 25 μg/ml (Fig. 3)

Immunoblotting results with the *S. typhi* OMP preparation showed that strong antibody binding was detected to all three major OMP bands. Also, we detected a significant reaction against a 53 kDa polypeptide (Fig. 4).

Discussion

In the serological diagnosis of TF there is clearly a need to develop a single, specific, sensitive, and rapid test to replace the Widal test which, despite its many limitations, is still widely used especially in developing countries[14]. In particular, there has been an increasing interest in the role played by OMPs as diagnostic antigens and as candidate antigens for vaccines[4-9,14]. The study described herein clearly shows that antibodies to OMP preparations are present in TF patient sera from two distant endemic areas. The OMP preparation used contain three major bands, that of OmpC, OmpF and OmpA and the SDS-PAGE profile of these bands are in agreement with those published previously[15].

The ELISA test showed high specificity and sensitivity; the mean absorbance values from TF sera (Group 1) was approximately 5.1 times higher than the mean absorbance of sera from TF-negative Groups, which included sera from healthy blood donors and from patients with other febrile illnesses common in the region. Suspected TF cases, with positive Widal titres (Group 2), rendered a lower mean absorbance value than TF-confirmed cases and higher dispersion in the absorbance values. Suspected TF cases, with negative Widal titres (Group 3), produced a mean absorbance value similar to healthy blood donors.

The test was positive in TF patients (Group 1) ranging from one week to one month of illness, and did not detect significant binding in normal sera from endemic areas. The response consisted mainly of IgG class antibodies (60%) with a minor IgM (20%) and IgA (12%) component. Individuals with suspected TF, and positive Widal titres, might not have rendered a positive blood culture due to antibiotic treatment, or improper specimen collection. Alternatively, if some of them are TF-negative, they

could be showing an anamnestic response. One individual with suspected TF, and negative Widal titre, gave a positive response in the TF-ELISA. Thus, the TF-ELISA might be detecting positive cases that are negative by Widal. This question warrants further investigation.

The TF-ELISA correlates very well with a positive blood/stool culture. In this respect it offers an advantage to the Widal test, where significant titres of anti-O and anti-H antibodies in normal sera often pose interpretation problems[1,2]. In addition, the initial study to develop this test[8,9] showed that it also has the capability of differentiating patients with other Gram-negative infections, such as those caused by enterotoxigenic *E. coli* (ETEC), *Campylobacter jejuni* and *Salmonella* Group "B"[8,9]. Consistent with this, is the finding that ELISA testing of sera from TF cases with OMP preparations from *E. coli* showed no cross-reactivity. Variable reactivity was, however, noted with OMP preparations extracted from other *Salmonella* spp.

There also appears to be reduced dispersion in absorbance values[8,9] in contrast with other ELISAs[16]. Our data confirms the results of earlier studies[7,8,9] and also suggests no apparent quantitative or qualitative differences in the reactivity of sera from Mexican or Malaysian patients with the OMP preparations.

The immunoblotting results with TF patient sera showed a significant reaction against OmpC, OmpF, and to a lesser extent against a 53 kDa band, and OmpA. Reaction to OmpC and OmpF is in agreement with that detected by Ortiz *et al*[17]. A 53 kDa band has been observed previously to be overexpressed under iron starvation[9], and might correspond to a 50-52 kDa protein(s) reported recently[18,19].

The present study also emphasizes the recent interest in the OMPs as important antigens in TF[14]. In relation to the wider use of OMPs in diagnostics there is a need for standardized methodology and more extensive field testing of OMPs as diagnostic antigens. It has been noted, for example, that various OMPs of different molecular weights are being detected in different parts of the world[14], and that technical differences in OMP preparation (e.g. heat treatment, contamination with flagellin, etc.) could account for such differences. It is not clear at present whether these various OMPs are similar or even identical; although the same results were obtained herein with OMP preparations from either a Mexican or a Malaysian strain of *S. typhi*. It should be noted that the OMP preparations used in the present study contain approximately 30% (w/w) LPS. In relation to this, it has been proposed that OMP-LPS complexes are involved in binding to antibodies from typhoid sera[6,8]. Other research groups have previously shown that OMPs of 50kDa or 52kDa are potentially useful as diagnostic antigens[18,19].

The fact that TF patient sera showed strong reactivity to OmpC also supports the suggestion of Mora *et al*[20], that this porin is expressed at significant levels in the human body during infection, and that it is exposed on the surface of the cell[21]. Further evaluation and field testing of OMP-based assays, including exchange of reagents, sera and test antigens between laboratories may eventually allow its more widespread use in areas endemic for TF.

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Table 1 ELISA testing of various sera with OMP-coated plates.

Group	Source of sera	Absorbance at 414 nm (Mean + S.D)
1	Typhoid fever (TF) (confirmed)	1.52 ± 0.23
2	Suspected TF (positive Widal)	1.28 ± 0.46
3	Suspected TF (negative Widal)	0.25 ± 0.19
4	Healthy donors	0.28 ± 0.10
5	Other fevers:	
	Leptospirosis	0.20 ± 0.08
	Typhus	0.24 ± 0.08
	Dengue	0.27 ± 0.08
6	Other infections	0.31 ± 0.16

Table II. Reactivity of sera from definitive TF cases with OMPs from different sources.

OMPs source	Positive/Tested*	Percentage
S. typhi	16/16	100
E. coli	0/16	0
S. paratyphi A	2/16	12.5
S. paratyphi B	8/16	50
S. typhimurium	9/16	56
S. anatum	4/16	25
S. aberdeen	0/16	0
S. worthington	0/16	0

*14 Malaysian and 2 Mexican sera from Group 1 were tested.

Control sera (Group 4) gave a baseline response.

Figure Legends

Fig. 1 SDS-PAGE profile of an OMP preparation from *S. typhi* grown in nutrient broth (lane a). The bands corresponding to OmpC, OmpF, and OmpA are indicated. Lane b contains the molecular weight markers.

Fig. 2 ELISA absorbance readings corresponding to various groups of sera.

Group 1: TF, blood and/or stool culture and Widal positive; Group 2: Suspected TF, blood culture negative, Widal positive; Group 3: Suspected TF, blood culture and Widal negative; Group 4: Healthy blood donors; Group 5: Leptospirosis, Rickettsial typhus, and Dengue fever; Group 6: Other infections (bacteremias by *S. enteritidis*, *S. paratyphi* B, and *Salmonella* spp., are indicated by an arrow in descending order).

Fig. 3 Inhibition-ELISA. Inhibition of antibody binding from typhoid fever (TF) patient sera to solid phase-bound *S. typhi* OMP preparations, by increasing concentrations (0.19-25 μg/ml) of free antigen. TF patient sera were diluted either 1:625 or 1:125, when used together with an OMP preparation or LPS as free antigen, respectively.

Fig. 4 Immunoblot of OMP preparations from *S. typhi* detected with sera from two TF patients (lanes a and b). Sera were diluted 1:400, and biotinylated goat anti-whole human serum was diluted 1:1,000. Molecular weight markers are indicated.

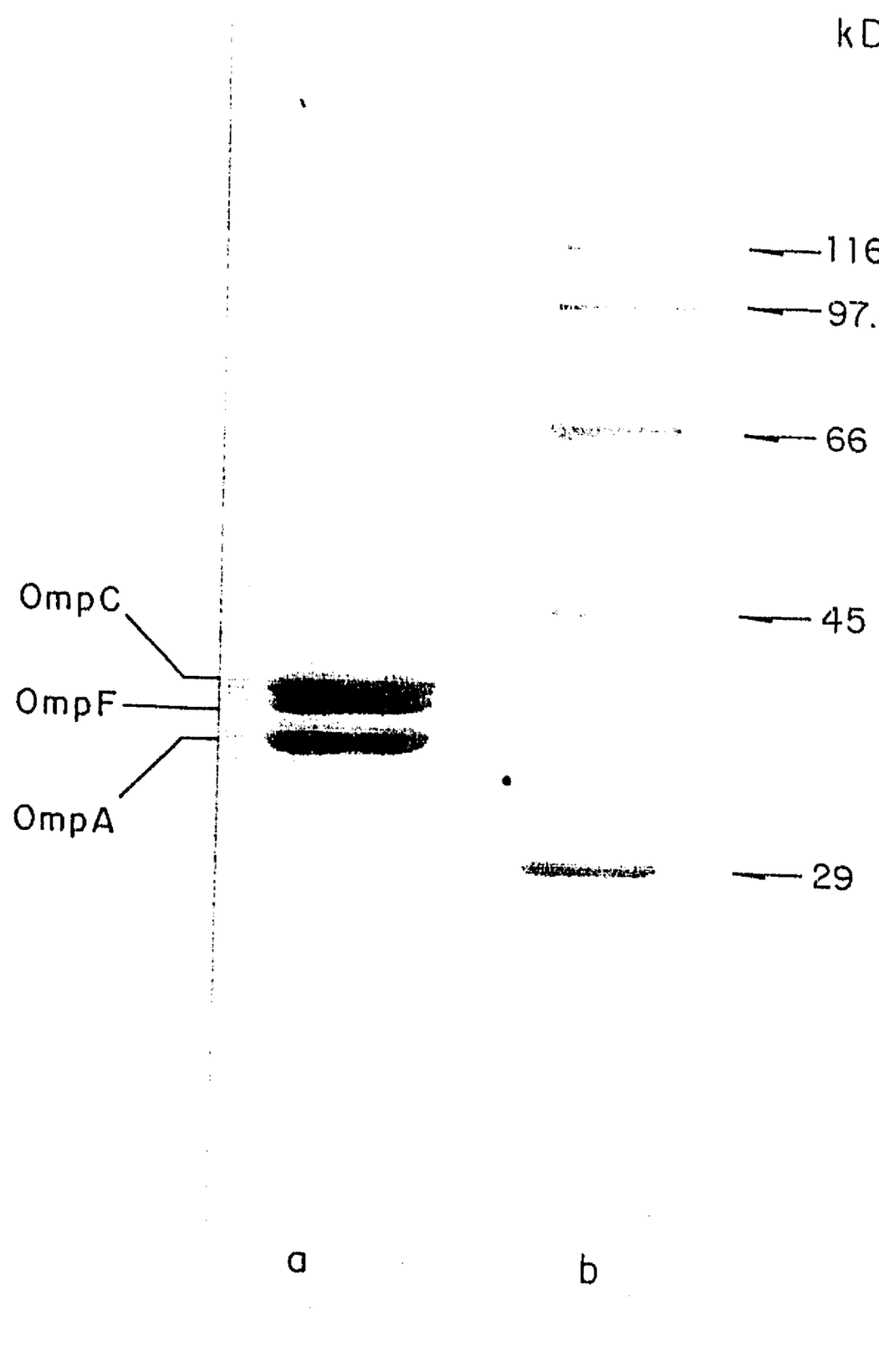
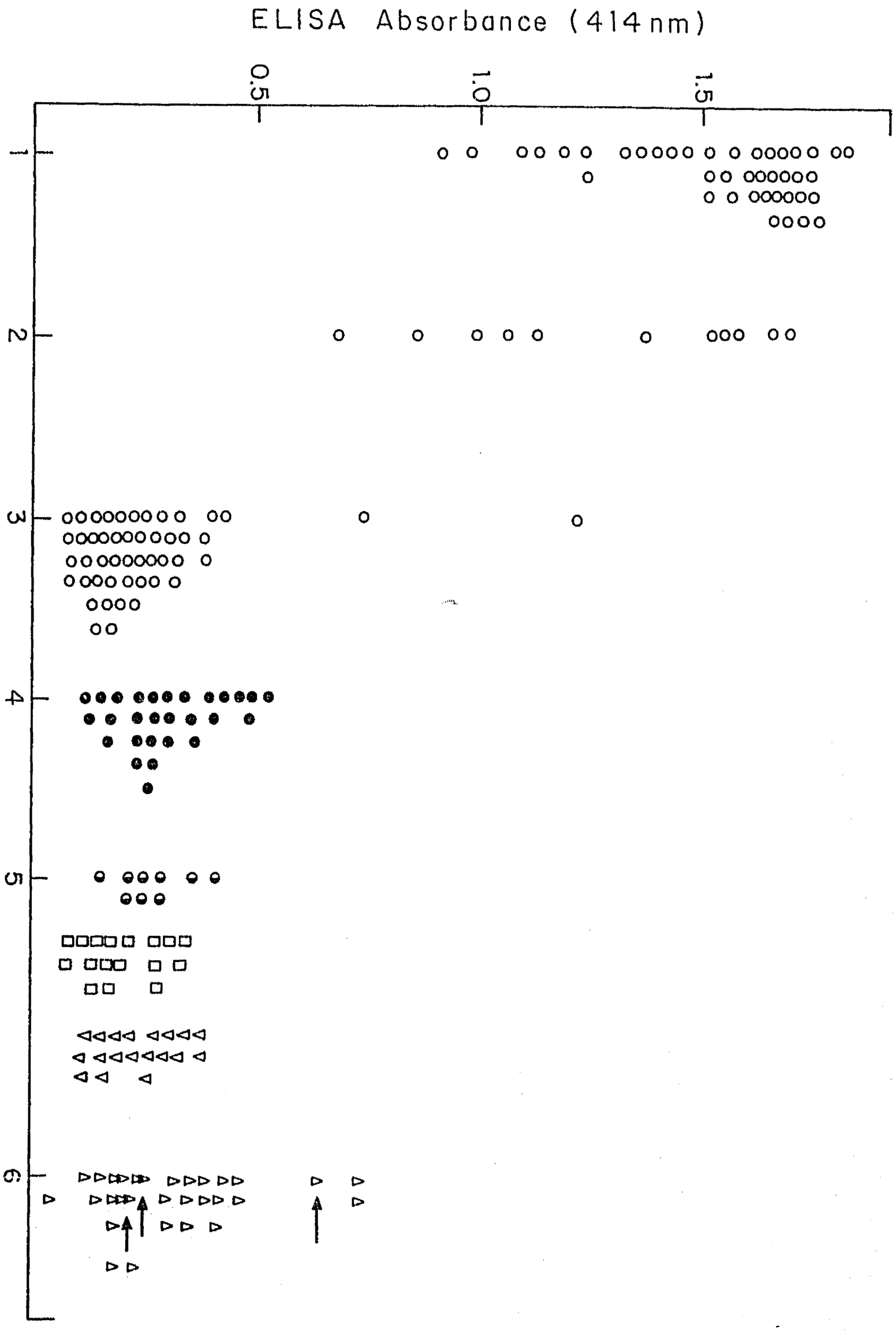


Figura 1

Figura 2



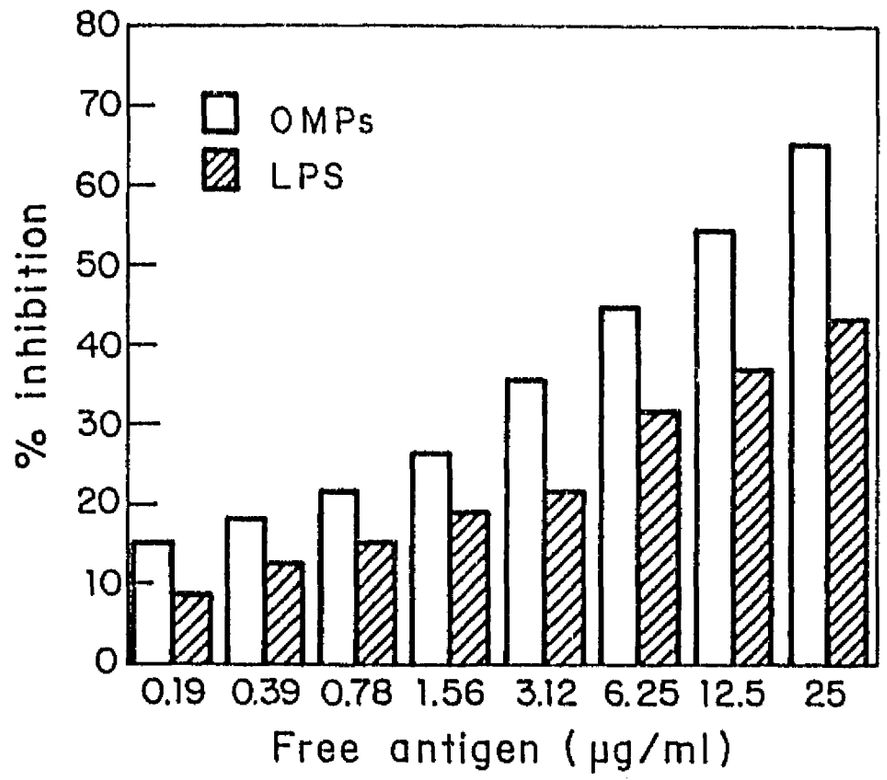

80
70
60
50
40
30
20
10
0
% inhibition
OMPs
LPS
0.19
0.39
0.78
1.56
3.12
6.25
12.5
25
Free antigen (µg/ml)

Figura 3

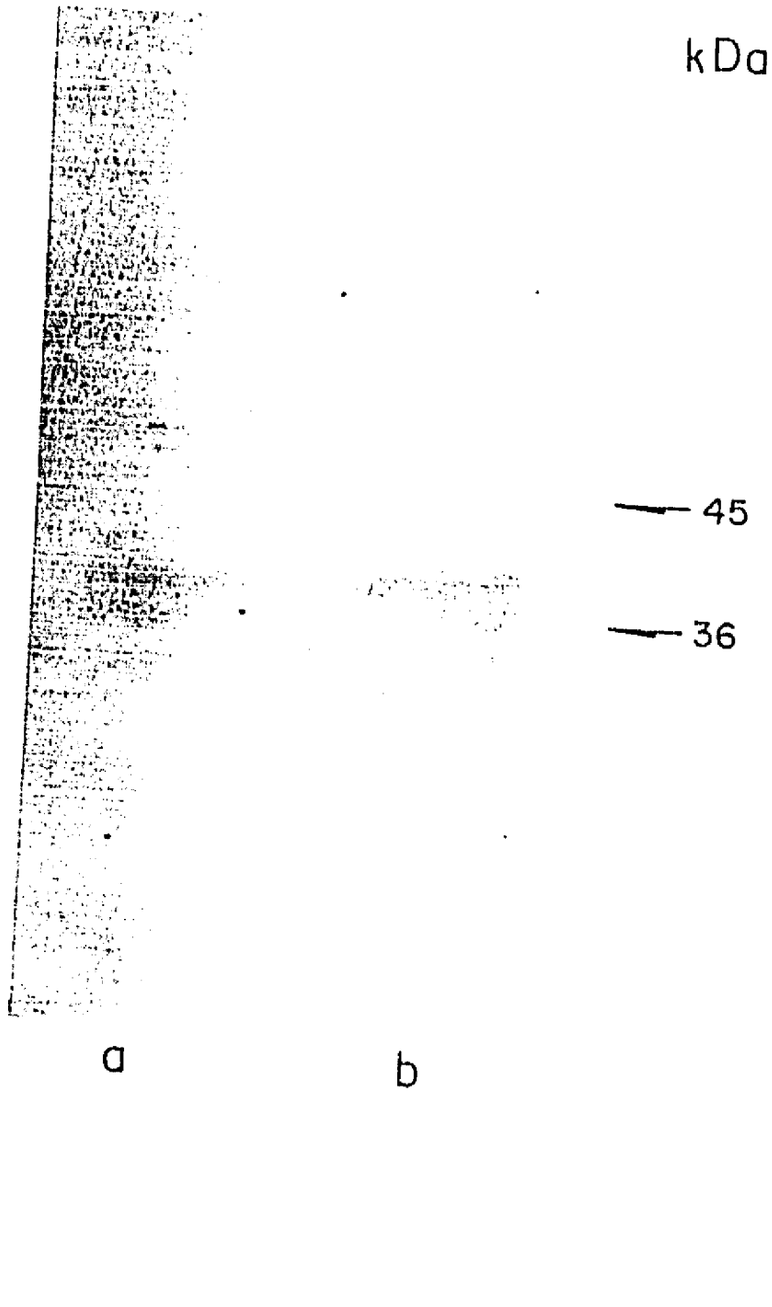


Figura 4

Apéndice III

Salmonella typhi OUTER MEMBRANE PROTEINS IN THE DIAGNOSIS OF TYPHOID FEVER

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ABSTRACT

An indirect ELISA was developed for the detection of specific human serum antibodies to *Salmonella typhi* outer membrane protein (OMP) preparations. Sera from fifteen individuals in each of the following groups were tested: a) acute phase (first week) typhoid fever (TF) patients, with positive *S. typhi* hemocultures; and controls which included sera from: b) diarrheic USA tourists infected with enterotoxigenic *Escherichia coli*; c) Mexican children (1-2 years old) with positive stool cultures for *Campylobacter jejuni*; d) adults with other Gram-negative bacteremia; and e) healthy blood donor Mexican adults. Results were obtained within five hours.

TF patient sera rendered absorbance readings, at 492 nm, of 1.4 U (SD 0.12); whereas control groups b), c), d), and e), had readings of 0.57 (SD 0.08), 0.55 (SD 0.16), 0.53 (SD 0.26), and 0.51 (SD 0.09), respectively.

OMP preparations typically contained 45 % (w/w) lipopolysaccharide (LPS) Preabsortion, and inhibition ELISAs with OMP and LPS preparations, indicate that protein or protein-LPS complex epitopes are involved in the specific humoral immune response during TF. In addition, the humoral response in rats immunized with OMP preparations is directed mainly against major OMPs, as evidenced by inhibition ELISA of rat sera using enriched *S. typhi* OmpC as antigen.

INTRODUCTION

Typhoid fever (TF) in man is the clinical manifestation of a systemic infection by *S. typhi*. Presently this disease is endemic in developing countries where sanitary conditions are suitable for the spread of TF, since its etiologic agent enters the body via the gastrointestinal tract, usually by ingestion of food or water contaminated by human feces. It has been estimated that anually, excluding China, 12.5 million persons suffer TF. The worldwide case-fatality is around 1 %[1].

At present, the single most effective method of diagnosis is the bone marrow culture, with an efficacy around 90 %; nevertheless, it is an aggresive and impractical procedure. Blood culture, more commonly used, has not shown as high sensitivity even when a set of up to three consecutive blood cultures are performed. However, with both methods, *S. typhi* isolation and identification takes at least 48 hours[1, 2]. One of the most used serodiagnostic methods is the Widal test, but it has low specificity in endemic areas, due to a high background of "O" lipopolysaccharide (LPS) side chain and "H" flagellar antibody titers in healthy adults[3]. Several research groups have reported new or improved diagnostic methods, including cultures[4], ELISA to detect antigens, i.e.: flagellin[5], proteins[6]; or to detect antibodies against proteic antigens[7, 8, 9]. The use of outer membrane proteins appears to increase the sensitivity of the ELISA[10]. However, in some of the reports there is higher variance in the readings than shown here, or there are not as many control groups.

We have designed an indirect ELISA to detect antibodies against *S. typhi* OMP preparations, using serum of early (first week of illness) TF patients. OMP preparations are from bacteria grown in an iron-starved medium, a typical condition of the blood and tissues[11].

RESULTS

OMP preparations.

S. typhi IMSS-1 cells were grown in nutrient broth (medium "A")[12], with and without 2.5 mg/ml of human or chicken egg transferrin. OMP preparations[13] were separated in SDS-PAGE gels, and three major proteins were observed: OmpC with 38.5 kDa[12, 14]; OmpF with 37.5 kDa[12, 15]; and OmpA with 34.5 kDa. In addition, we observed several minor polypeptides of: 18, 26, 30, 45, 57 (flagellin) and 62.5 kDa; and a set of polypeptides overexpressed under iron-starvation of: 53, 72, 77, 82, 92, 97.5 and 103 kDa bands. These OMP preparations contained 45 % LPS (w/w), determined as described previously[16].

TF-ELISA

General procedures were as described previously[17]: ELISA microplates were coated with OMP preparations obtained from bacteria grown under iron-starvation at a final protein concentration of 5 µg/ml diluted in PBS/B-1[18] pH 7.4. The optimal sera dilution was the reciprocal of 3 125. Anti-human horseradish peroxidase conjugated immunoglobulins, and orthophenylenediamine-H_2O_2 enzyme sustrate were as described previously. Readings were at 492 nm. Human sera were collected at the Instituto Nacional de la Nutrición "Salvador Zubirán" and were grouped as follows:

Group I. Typhoid fever: Mexican adults (18-45 years old, age matched with Group V), with a positive *S. typhi* blood cultures; Group II. Adult USA travelers with acute diarrhea and positive stool cultures for enterotoxigenic *E. coli*; Group III. Mexican children (1-2 years old) with *C. jejuni* in stool cultures; Group IV. Healthy Mexican adult blood donors;

and Group V. Mexican adults with other bacteremias. Each group consisted of 15 individuals. Each specimen was tested twice by duplicate (see Table I).

Table I

GROUP	MEAN	S. D.
I	1.41	0.122
II	0.57	0.084
III	0.55	0.042
IV	0.51	0.089
V	0.53	0.260

Inhibition OMP-ELISA.

The capability of OMP preparations to inhibit binding of the antibodies against OMP preparation to solid phase-bound OMP preparations or commercial protein free-LPS (LPSs), was assayed by an inhibition ELISA method. A 50 % inhibition of the antibody binding was observed with aproximately 18 µg/ml or even less of free OMP preparations, whereas free LPSs did not inhibit 50 % even with 50 µg/ml.

Also, inhibition OMP-ELISA was performed with rat-antisera against OMP preparations from the following strains: *S. typhi*, *S. typhimurium*, *S. arizonae*, *S. anatum* and *S. aberdeen*. We detected a 50 % inhibition of antibody binding to solid phase-bound OMP preparations with 25 µg/ml of homologous antigen, whereas with 50 µg/ml of LPSp the inhibition of binding was only 12-25 %.

In order to assess the specificity of antibodies against OMPs or LPS in another manner, we tested rat antisera against an OMP preparation from *E. coli* HB101 (a strain with a length-defective LPS), carrying a cloned *S. typhi ompC* gene in pVF27. We detected 50 % inhibition of antibody binding to solid phase-bound homologous antigen with 4 µg/ml of free antigen. In contrast, 6.25, 7.5 and 12.5 µg/ml were necessary to inhibit, in a similar manner, the binding of heterologous antisera against OMP preparations from *S. typhi* IMSS-1 (a smooth strain), and from *E. coli* harboring or not cloned *E. coli ompC* in pMY111[19].

DISCUSSION

Developing countries usually have shown a high incidence of TF as they are endemic areas, and an early and efficient diagnosis is needed. In contrast, in developed countries where *Salmonella* infections are not common, the use of the Widal Test is enough, while in endemic areas it has a low predictive value[3, 20]. We tested *S. typhi* OMP preparations as antigen in an ELISA system, and we designated it as TF-ELISA, that could be useful in early diagnosis in

an endemic area. TF-ELISA has shown high specificity (100 %) and sensitivity (100 %), as compared with blood culture, because TF patient sera tested were chosen on the basis of *S. typhi* isolation.

The arithmetic mean of TF-ELISA absorbance values from the TF patient group was 2.6 times higher than the mean value averages from the TF-negative groups.

An important aspect observed with our results is the reduced dispersion in the absorbance values, in contrast with other reported ELISA[6]. Additional important features were the capability of differentiating patients with other bacteremia and the reduced performing time (4.5 hours), as compared with blood isolation and identification of the bacteria.

Based on the described results, OMP preparations in TF-ELISA could be a potential system to be used routinely in endemic areas. However, it needs to be tested in field trials, and with other groups like chronic carriers, TF vaccinated people and children under 2 years.

Previously, several research groups have demonstrated the importance of OMPs, in protection against *Salmonella* infection with animal models[21, 22]. In order to evaluate the importance of OMPs and LPS (45 % w/w of OMP preparations) in the detected humoral immune response against OMP preparations, we tested some sera in inhibition OMP-ELISA, and we observed that OMPs are the major component recognized by antibodies from TF-patients. However, the presence of LPS in these OMP preparations appears to be important.

AKNOWLEDGEMENTS

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Apéndice IV

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Expression of *Salmonella typhi* and *Escherichia coli* OmpC is influenced differently by medium osmolarity; dependence on *Escherichia coli* OmpR

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Summary

OmpC, a major outer-membrane protein, is highly expressed when *Salmonella typhi* is grown in nutrient broth (NB) of either low (NB + 0% sucrose) or high (NB + 20% sucrose) osmolarity. This contrasts with the expression of *Escherichia coli* OmpC, which is inhibited in low osmolarity and enhanced in high osmolarity, as has been described previously (van Alphen and Lugtenberg, 1977; Verhoef *et al.*, 1979; Kawaji *et al.*, 1979). Nevertheless, expression of *S. typhi* OmpC is dependent on the *E. coli* OmpR transcriptional activator. These findings suggest differences between the mechanisms of osmoregulation of gene expression in both bacteria, although common effectors appear to be shared.

Introduction

Salmonella typhi is the causal agent of typhoid fever (TF) in humans. It is estimated that more than 12 million human beings (excluding the population of China) are affected annually by this disease. The case-fatality rate is 1% (Edelman and Levine, 1986). *S. typhi* is usually acquired by oral infection of contaminated food or beverages. The bacteria adhere to and penetrate the intestinal epithelium; they are then ingested by macrophages and transported to the reticuloendothelial system, where they multiply and propagate throughout the organism (Finlay and Falkow, 1989). The study of different aspects of TF, ranging from molecular biology to epidemiology, offers not only the opportunity of making an impact on health biotechnology, through the development of new vaccines and diagnostic methods, but also of acquiring an insight into basic biological processes, such as the genetic expression of pathogenic determinants (Calva *et al.*, 1988).

Outer-membrane protein (OMP) preparations have elicited active immunity in mice against *Salmonella typhimurium* and *S. typhi* (Udhayakumar and Muthukkaruppan, 1987a,b; Isibasi *et al.*, 1988), and the presence of specific anti-OMP antibodies in the sera of patients might be of diagnostic value (Calderón *et al.*, 1986; A. Verdugo-Rodríguez *et al.*, unpublished). These observations have led us to study the molecular structure and function of *S. typhi* OMPs and of their respective genes. Thus, we have previously reported the isolation and characterization of the *ompC* gene which codes for OmpC, a major outer-membrane protein (Puente *et al.*, 1987; 1989).

Escherichia coli ompC has been extensively studied by other authors, who have reported that it is expressed preferentially in media of high osmolarity and is repressed in low osmolarity. In contrast, *E. coli ompF* is highly expressed in low osmolarity and is repressed in high osmolarity (Forst and Inouye, 1988).

ompC and *ompF* code for *E. coli* porins OmpC and OmpF, respectively; there are approximately 10^5 porin molecules per cell, forming non-selective pores through which small hydrophilic molecules traverse the outer membrane. Even though the relative proportions of both proteins vary depending on growth conditions, the sum of their quantities tends to remain constant (Nikaido and Vaara, 1985). The synthesis of these porins is regulated by the products of the genes *envZ* and *ompR*. Protein EnvZ acts as an osmosensor, transmitting information to the OmpR protein, which in turn acts as receptor of the information, regulating (activating) transcription of *ompC* or *ompF* (Gross *et al.*, 1989).

It has been observed recently that expression of *E. coli ompC* is repressed in low osmolarity only when the cultures are well aerated, i.e. it is strongly induced in anaerobiosis. Conversely, expression of *ompF* was relatively unaffected by low availability of oxygen. Anaerobic induction of *S. typhimurium* OmpC was similar to that observed for *E. coli ompC* (Ni Bhriain *et al.*, 1989).

We have previously described the *S. typhi ompC* gene and found that it is 77% homologous to its *E. coli* counterpart at nucleotide level. The respective derived

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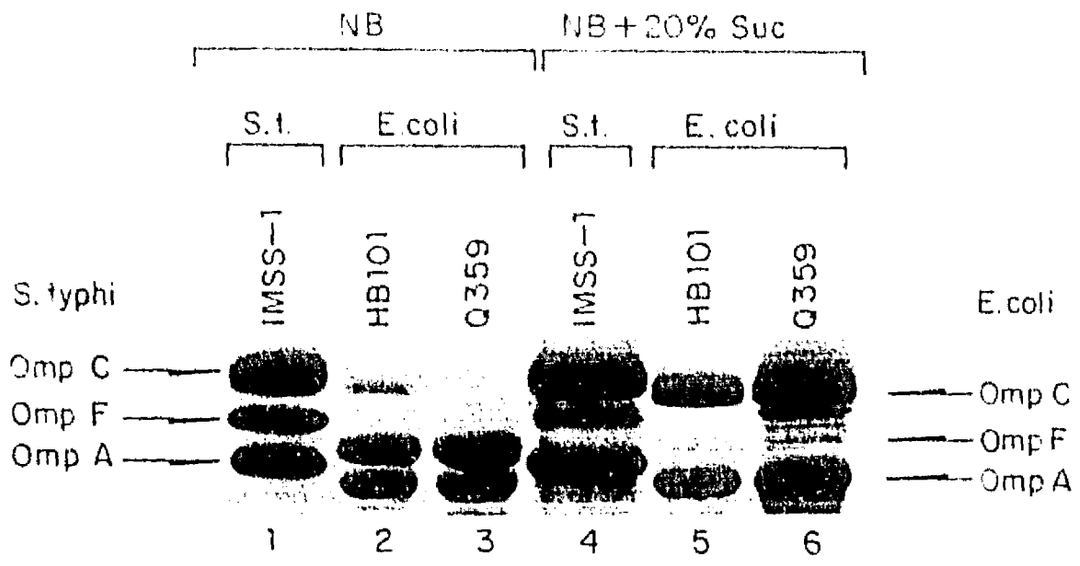


Fig. 1. Electrophoretic pattern of OMP preparations from *S. typhi* IMSS-1 (lanes 1 and 4), from *E. coli* HB101 (lanes 2 and 5), and from *E. coli* Q359 (lanes 3 and 6), grown in low osmolarity (NB; lanes 1–3) or high osmolarity (NB + 20% sucrose; lanes 4–6). The positions of the *S. typhi* and *E. coli* OMPs are shown in the left and right margins, respectively.

OmpC amino acid sequences are predicted to be 79% similar. The nucleotides at positions −1 to −194, upstream of the putative *N*-terminal ATG of *S. typhi ompC*, share 91% similarity with the corresponding region in *E. coli*. This region contains three putative promoters and part of a possible binding site for OmpR. In contrast, the region from nucleotides −195 to −333 shows only 61% similarity between both bacteria (Puente *et al.*, 1987; 1989). Here we report the expression of OmpC in *S. typhi* under low and high osmolarity conditions. We also observed the lack of expression of the *S. typhi ompC* gene in an *E. coli* strain mutated for the OmpR transcriptional activator.

Results

Synthesis of S. typhi *OmpC in low and high osmolarity*

During analysis of the electrophoretic patterns of outer-membrane protein (OMP) preparations, *S. typhi* OmpC, encoded by its corresponding gene cloned in *E. coli* plasmid pVF27, comigrated with an endogenous *S. typhi* OMP both in low (NB + 0% sucrose) and high osmolarity culture conditions (NB + 20% sucrose) (Puente *et al.*, 1987).

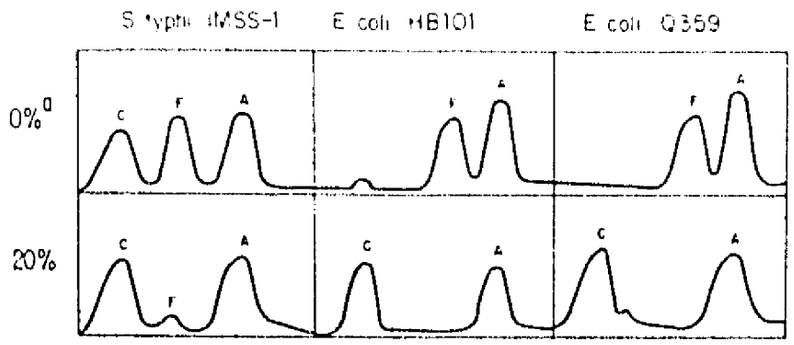
The expression of major OMPs from *S. typhi* reference strains IMSS-1 and Ty2, and from MK clinical isolates, was compared with that of *E. coli* HB101 and Q359 (two laboratory strains derived from K12). This was done in order to eliminate the possibility that the initial observation on *S. typhi* OmpC expression was due to some peculiarity of the culture conditions or of the strains used. Expression of *E. coli* OmpC was used as control since its osmoregulation is extensively documented (Forst and Inouye, 1988).

Figure 1, depicting electrophoretic patterns of major OMPs, is a representative example of the analysis performed on the various *S. typhi* strains. *S. typhi* OmpC, from strain IMSS-1, was expressed at the same level both at low (lane 1; NB) and high (lane 4; NB + 20% or 0.57 M sucrose) osmolarity. This phenomenon was also observed in strain Ty2 and in eight independent MK clinical isolates. In contrast, expression of *E. coli* OmpC was repressed in low osmolarity (lanes 2 and 3; NB) and favoured in high osmolarity (lanes 5 and 6; NB + 20% sucrose), in accord with previous observations (van Alphen and Lugtenberg, 1977; Verhoef *et al.*, 1979; Kawaji *et al.*, 1979). Furthermore, the same results were obtained regardless of whether the growth temperature was 4, 20, 37 or 42°C (not shown).

The osmolarity of NB was 176 mOsm. In comparison, the osmolarity of a low-osmolar K medium (Kennedy, 1982) of M9 salts plus casamino acids (Maniatis *et al.*, 1982), and of LB-rich medium (Maniatis *et al.*, 1982) was 125, 270, and 448 mOsm, respectively. The identity of the other OMP bands in Fig. 1, in both *S. typhi* and *E. coli*, was assigned as follows: OmpF was assigned because its synthesis was repressed in high osmolarity; OmpA because it showed, as in *E. coli* (Schnaitman, 1974), heat-modifiable electrophoretic migration and constitutive synthesis (not shown); and *E. coli* OmpC because of the expression of plasmid pMY111 (Mizuno *et al.*, 1983) harbouring *E. coli ompC*.

The apparent molecular weights of the three main *S. typhi* OMPs appeared greater than the corresponding ones in *E. coli*. In fact, *S. typhi* OmpC is 11 amino acids longer than *E. coli* OmpC, by comparison with the amino acid sequences derived from the corresponding *ompC* nucleotide sequences (Puente *et al.*, 1989). It is interesting to note that the *S. typhi* OMP profiles did not seem to contain a fourth major band, in the 35–45 kDa range, equivalent to *S. typhimurium* OmpD (Bennet and Rothfield, 1976; Nurminen *et al.*, 1976), an OMP that has been recently reported to be associated with virulence (Dorman *et al.*, 1989).

Figure 2 shows a quantitative densitometric analysis of OMP expression taken from Fig. 1. A clear assessment



	S. typhi IMSS-1		E coli HB101		E. coli Q359	
	0%[a]	20%	0%	20%	0%	20%
OmpC	0.86[b]	0.82	0.08	1.10	—	0.97
OmpF	0.84	0.14	0.91	—	0.91	—
OmpA[c]	1.00	1.00	1.00	1.00	1.00	1.00

Fig. 2. Densitometric scans of the OMP electrophoretic patterns from *S. typhi* IMSS-1, and *E. coli* HB101 and Q359 grown in low (NB + 0% sucrose) (a) or high (NB + 20% sucrose) osmolarity. The upper portion shows the graphs obtained from the scans. The lower portion shows the relative areas of the absorbance peaks (b), corresponding to OmpC (C), OmpF (F), and OmpA (A), taking OmpA in each lane as unit (c). The data are the representative averages of at least three different experiments.

can be made of the constitutive synthesis of *S. typhi* OmpC in low (NB + 0% sucrose) and high (NB + 20% sucrose) osmolarity, as well as of the repression of *E. coli* OmpC synthesis in low osmolarity relative to high osmolarity. The synthesis of OmpF in both bacteria was regulated in a similar manner. In addition, the total amount of OmpC plus OmpF in both conditions was almost constant for *E. coli*, in contrast with what is observed in *S. typhi* where this amount was higher in low osmolarity.

The data depicted in the figure did not exclude the possibility that the band that we had identified as OmpC, when synthesized in *S. typhi* at low osmolarity, was not in fact OmpC, but another protein with the same electrophoretic mobility. This was examined by comparing the peptide map of this protein (Fig. 3, lane 4) with that from the *S. typhi* OmpC protein, synthesized from the cloned gene in *E. coli* (Fig. 3, lane 5). The electrophoretic profiles of the peptides obtained upon partial digestion with *Staphylococcus aureus* protease V8 was identical for both proteins, in contrast with different profiles obtained for *E. coli* OmpC, synthesized from the cloned multicopy gene (Fig. 3, lane 3), or for *S. typhi* OmpF (Fig. 3, lane 2), both being synthesized in low osmolarity. Furthermore, the profile in lane 4 (Fig. 3) seems to correspond only to OmpC, which eliminates the possibility that two major proteins are superimposed in the same band.

The profile in lane 5 (Fig. 3) shows no major contamination with *E. coli* OmpC, reflecting inhibition of the synthesis of endogenous OMPs when cloned multicopy *omp* genes are expressed, as has been observed previously (Click *et al.*, 1988). Furthermore, the profiles of OmpC synthesized in high osmolarity by *S. typhi* and by *E. coli* are identical to those shown in lanes 5 and 3 (Fig. 3), respectively (not shown).

Synthesis of S. typhi *OmpC is OmpR-dependent*

In order to determine if synthesis of *S. typhi* OmpC in *E. coli* required the OmpR transcriptional activator, the *S. typhi* and the *E. coli ompC* genes, contained in plasmids pVF27 and pMY111, respectively, were introduced into *E. coli* KY2562. This strain lacks a functional OmpR, because of a 19-amino-acid deletion, which confers on the bacteria an OmpC-minus, OmpF-minus phenotype (Nara *et al.*, 1986).

Figure 4 shows that both *S. typhi* (KY2562/pVF27) and *E. coli* (KY2562/pMY111) OmpC were not expressed in an OmpR-minus background. In contrast, overexpression of the multicopy cloned *ompC* genes in strain HB101 (OmpR-plus), used as a positive control, resulted in the inhibition of endogenous OMP synthesis, as has been described previously (Click *et al.*, 1988). The identity of the *S. typhi* OmpC protein, in the pattern corresponding to HB101/pVF27, was determined by analysis of peptide maps, as in the experiment described in Fig. 3. These results indicate that some features of the mechanism of *ompC* expression such as dependence on OmpR, appear to be shared by both bacteria.

Discussion

We show here that there is a different effect of medium osmolarity on the expression of *S. typhi ompC* relative to *E. coli ompC*. Since expression of *E. coli ompC* is favoured at higher osmolarity and growth temperatures (van Alphen and Lugtenberg, 1977; Verhoef *et al.*, 1979; Kawaji *et al.*,

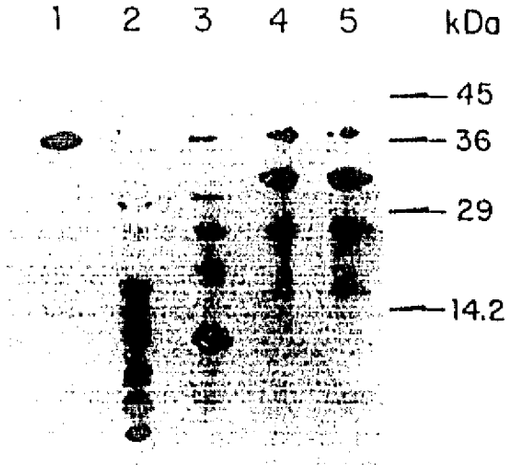


Fig. 3. Electrophoretic pattern of the peptide maps obtained by partial digestion with *S. aureus* V8 protease of *S. typhi* OmpF synthesized in low osmolarity (lane 2), *E. coli* OmpC synthesized in low osmolarity from the cloned gene in pMY111 (lane 3), *S. typhi* OmpC synthesized in low osmolarity (lane 4), and *S. typhi* OmpC synthesized under low osmolarity in *E. coli* from the cloned gene in pVF27 (lane 5). *E. coli* OmpC was included as an undigested control (lane 1).

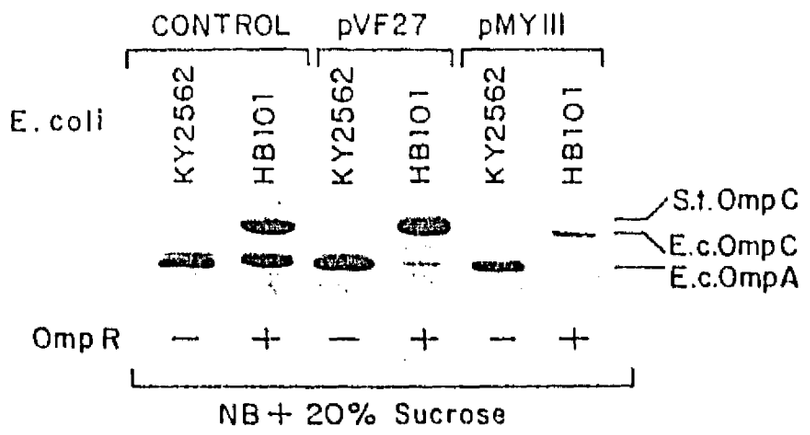


Fig. 4. Electrophoretic pattern of OMP preparations from *E. coli* KY2562 (OmpR-minus) and HB101 (OmpR-plus), without a plasmid (controls), or containing either plasmid pVF27 (harbouring *S. typhi ompC*) or pMY111 (harbouring *E. coli ompC*). The bacteria were grown in NB + 20% sucrose.

1979; Lugtenberg *et al.*, 1986), it has been speculated that this could be significant when the bacterium is growing inside a host organism, in contrast to when it is growing in an external environmental niche, usually of lower osmolarity and temperature (Nikaido and Vaara, 1985). This concept contrasts with our findings, where we could not observe lowering of the expression of *S. typhi ompC* at low osmolarity.

Studies with *E. coli ompC–lac* fusions have shown that there is a transient 3.4-fold increase in the rate of expression for *ompC*, after a 10 min lag, upon shift from low to high osmolarity (Jovanovich *et al.*, 1988). In addition, it has been shown that expression of *E. coli* and *S. typhimurium ompC* is increased in anaerobiosis, with an overlap between the responses to anaerobic and osmotic stress, where DNA supercoiling may play an important role (Ni Bhriain *et al.*, 1989; Graeme-Cook *et al.*, 1989). Similar experiments with *S. typhi ompC* should shed more information on the role of these and other factors, including the carbon source (Scott and Harwood, 1980), on gene expression.

Most of the nucleotide sequence differences in the 5′ upstream regulatory region, between *S. typhi* and *E. coli ompC*, are contained in the more distal −195 to −333 portion (Puente *et al.*, 1989), and such nucleotides could be responsible for the differential expression of both genes. In particular, a putative OmpR-binding site differs in five out of twenty six residues. In the −1 to −333 portion, there are three putative Pribnow boxes which are identical in both bacteria, so they appear not to be involved in the different responses of the *ompC* genes to osmolarity. It has been shown that a single-base mutation in the most proximal Pribnow box of the *E. coli* gene results in a high OmpR-dependent constitutive level of expression (Ozawa *et al.*, 1987).

We have reported here that expression of *S. typhi ompC* in *E. coli* is OmpR-dependent. Whether *S. typhi* possesses an EnvZ/OmpR-like signal-transducing sensor/regulator system that functions in a different manner as the corresponding *E. coli* system does, is an open question. Interestingly, in *S. typhimurium*, mutations in *ompR* result in attenuated virulence (Dorman *et al.*, 1989), and avirulent mutants sensitive to macrophage defensins map in the PhoP/PhoQ sensor/regulator system (Miller *et al.*, 1989). Furthermore, it has been observed that *S. typhimurium invA*, which allows penetration of tissue-culture cells and has a role in the invasion of Peyer's patches and small-intestine cell wall, is expressed at an eight-fold higher level upon shift from low to high osmolarity: this phenomenon is influenced by changes in DNA supercoiling (Galán and Curtiss, 1990). However, further study will be required to determine if our observations indicate the existence of a specialized global genetic regulatory circuit and to determine its role in the pathogenicity of *S. typhi*.

Experimental procedures

Bacterial strains and plasmids

The *S. typhi* strains (serotypes 9, 12, d and Vi) were the following: IMSS-1, isolated from a patient with TF, generously donated by Dr Jesús Kumate and collaborators (Instituto Mexicano del Seguro Social, México DF) (Isibasi *et al.*, 1988); Ty2, obtained from the American Type Culture Collection (ATCC No. 19430); and eight clinical isolates of the MK series, isolated from TF patients with different clinical symptoms, generously made available by Dr Guillermo Ruiz-Palacios and collaborators (Instituto Nacional de la Nutrición 'Salvador Zubirán', México DF). The *E. coli* strains were HB101, obtained from and described by Boyer and Roull-and-Dussoix (1969); Q359, obtained from and described by Karn *et al.* (1980); and KY2562, obtained from and described by Nara *et al.* (1986).

Plasmid pVF27, containing *S. typhi ompC* (Puente *et al.*, 1987), was constructed in our laboratory. Plasmid pMY111, containing *E. coli ompC* (Mizuno *et al.*, 1983), was kindly made available by Dr M. Inouye from The University of Medicine and Dentistry of New Jersey, USA.

Bacterial growth

Bacteria were grown in Nutrient Broth (Bacto Nutrient broth; NB; Difco) at 37°C with vigorous agitation (250 r.p.m.) in flasks at least 10 times the size of the culture, up to late logarithmic phase. Low and high osmolarity growth was when the media were prepared without (0%) or with (20%) added sucrose, respectively.

Osmolarity determinations

The osmolarity of the various culture media was determined in an Osmette precision Osmometer (model no. 2007; Precision Systems, Inc.). The values reported were the averages of two readings, which varied by less than 3%.

OMP preparations

The Triton X-100 insoluble membrane fractions were obtained according to Matsuyama *et al.* (1984).

Polyacrylamide gel electrophoresis of OMP preparations

OMP preparations were subjected to electrophoresis (5h at 20mA) in polyacrylamide slab (14cm × 10cm × 1.5mm-thick) gels prepared with 11% acrylamide, 0.3% bis-acrylamide, 0.1% sodium dodecyl sulphate (SDS), 8M urea, and the discontinuous buffer system of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue.

Densitometric analysis of OMP electrophoretic patterns

Scans were performed on picture negatives of Coomassie Brilliant Blue-stained OMP electrophoretic patterns with a laser beam densitometer from Biomed Instruments, Inc.

Peptide maps

The procedure for obtaining peptide maps was based on that described by Cleveland *et al.* (1977). Briefly, the bands of interest from an OMP electrophoretic profile were cut out of a dry gel previously stained with Coomassie Brilliant Blue. They were incubated with 0.1 units of *S. aureus* V8 protease (Sigma Chemical Co.) per μg of protein, for 30 min at 25°C. The resulting peptides were separated by electrophoresis (20mA, 4h) in polyacrylamide (15% acrylamide, 0.4% bis-acrylamide, 0.1% SDS) slab (14cm × 10cm × 1.5mm-thick) gels in the discontinuous buffer system (Laemmli, 1970). They were visualized by staining with Coomassie Brilliant Blue.

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