DEPARTAMENTO DE MICROBIOLOGÍA Y ECOLOGÍA

ESTUDIOS SOBRE LA INMUNOGENICIDAD Y LOS MECANISMOS FISIOPATOLÓGICOS DE LA PROTEÍNA NSP4 DE ROTAVIRUS

JESÚS RODRÍGUEZ DÍAZ

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Estudios sobre la inmunogenicidad y los mecanismos fisiopatológicos de la proteína NSP4 de rotavirus

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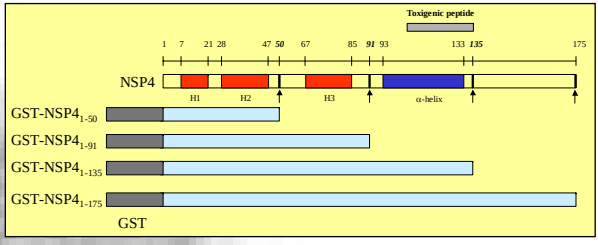

Tesis Doctoral

VNIVERSITAT DE VALÈNCIA

Departament de Microbiologia i Ecologia
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HACE CONSTAR

que el presente trabajo de investigación titulado **"Estudios sobre la inmunogenicidad y los mecanismos fisiopatológicos de la proteína NSP4 de rotavirus"**, presentado como recopilación de publicaciones, ha sido realizado bajo mi dirección por **Jesús Rodríguez Díaz**, licenciado en Biología, para optar al título de **Doctor en Biología,** dentro del Programa de Doctorado de **"Microbiología"** (694-275D) de la Universitat de Valencia.

En Valencia, a 7 de febrero de 2005

Prof. Dr. Javier Buesa Gómez

"Siempre que enseñes, enseña a la vez a dudar de lo que enseñas".

(José Ortega y Gasset)

La lógica es un método sistemático para llegar

con confianza a la conclusión errónea.

(Máxima de Manly)

AGRADECIMIENTOS

Hace más de cuatro años que comenzó mi andadura por el mundo de la Virología; en este tiempo muchas son las personas que han influido positivamente a que este proyecto vea la luz. En primer lugar quiero agradecer a mis padres y hermanos todo el apoyo prestado, no sólo durante el periodo que ha durado la elaboración de esta tesis, sino tambien por la familia tan maravillosa que me ha tocado en suerte. A mi director Javier Buesa todo el apoyo prestado y la oportunidad brindada, sin la cual esta tesis hubiera sido imposible. A todos los compañeros del laboratorio de Virología del Departamento de Microbiología de la Facultad de Medicina de Valencia, donde se realizó la mayor parte de esta tesis: Pilar, Ana, Beatriz, Rebeca, Rima y los últimos en llegar: Clara y Juanma; ha sido un verdadero placer haber podido trabajar con vosotros y compartir no sólo las experiencias del laboratorio.

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INTRODUCCIÓN GENERAL

1. Breve historia y situación taxonómica de rotavirus

En 1973 Bishop y colaboradores describieron un virus en forma de rueda tras la observación al microscopio electrónico de cortes de intestino delgado procedentes de niños con gastroenteritis (Bishop et al., 1973). No obstante diez años antes, en 1963, se habían encontrado en la mucosa intestinal de ratones con diarrea unos virus con una morfología similar. El virus aislado de los ratones fue llamado *virus de la diarrea epizoótica de ratones lactantes* (EDIM) (Adams y Kraft, 1963). En ese mismo año, Malherbe y Harwin (1963) aislaron partículas víricas a partir del tejido rectal de un mono 'vervet' sano. El virus fue llamado SA11, del inglés "*simian agent* 11". Este aislado pudo ser propagado en una línea celular procedente de la misma especie de simio. Pocos años después Mebus y colaboradores demostraron la presencia de otro virus de tamaño y morfología similares a los descritos anteriormente en heces de terneros con diarrea (Mebus et al., 1969). El virus aislado por Mebus también pudo ser propagado en células fetales bovinas (Mebus et al., 1971).

Debido a su morfología en forma de rueda, estos nuevos virus causantes de gastroenteritis fueron llamados rotavirus, del término en latín "*rota*" que significa rueda.

Finalmente, en 1979, Mathews estableció el género rotavirus e incorporó este nuevo género a la familia *Reoviridae*. Esta familia está compuesta por virus de estructura icosaédrica sin envoltura lipídica cuya cápside está formada por capas concéntricas de proteínas con un diámetro de entre 60 y 80 nm. El genoma está compuesto por ARN de doble cadena (ARNbc) que se encuentra dividido en 10 a 12 segmentos, lo cual permite que ocurran recombinaciones y reorganizaciones genéticas. La replicación de los virus pertenecientes a la familia *Reoviridae* ocurre en el citoplasma celular, en unas estructuras subcelulares ricas en proteínas víricas llamadas viroplasma.

Dentro de la familia *Reoviridae* se agrupan seis géneros diferentes: Orthoreovirus, Orbivirus, Rotavirus, Coltivirus, Aquareovirus, Cypovirus, Fijivirus, Phytoreovirus y Oryzavirus. Los cuatro primero géneros infectan mamíferos y aves.

2. Características generales de los rotavirus

Los rotavirus son virus no envueltos con morfología icosaédrica y un tamaño que oscila entre 60 y 80 nm. La cápside está formada por tres capas proteicas concéntricas. La capa más interna forma el core que engloba el genoma vírico y los enzimas encargados de la replicación del ARN.

El genoma de rotavirus está compuesto por 11 segmentos de ARNbc, numerados del 1 al 11 tras su separación electroforética. Cada segmento codifica al menos para una proteína. Debido a que las células eucarióticas no poseen ningún enzima ARN polimerasa ARN dependiente, los segmentos del genoma de rotavirus no son infectivos en ausencia de la ARN polimerasa viral. Una de las principales características de rotavirus y debido a la presencia de 11 segmentos genéticos es el proceso de *"reassorting"*: cuando una misma célula es infectada por más de una cepa de rotavirus éstas pueden intercambiar fragmentos genéticos, dando lugar a nuevas cepas víricas.

La infectividad de rotavirus necesita de la activación por proteasas. Los rotavirus humanos han sido propagados clásicamente en la línea celular MA104, tras su activación con tripsina y añadiendo tripsina al medio de cultivo (Estes et al., 2001, Wyatt et al., 1983). Los rotavirus, al igual que el resto de representantes de la familia *Reoviridae*, replican en el citoplasma y pueden formar cuerpos de inclusión compuestos por proteínas víricas cristalizadas. Otra característica de los rotavirus durante su morfogénesis es el estado transitorio en el que las partículas virales se encuentran envueltas. La liberación de la progenie vírica ocurre por lisis celular o mediante transporte vesicular no convencional en células epiteliales polarizadas.

3. Clasificación de rotavirus

Los rotavirus se clasifican en serogrupos, subgrupos y serotipos según sus características antigénicas. En base a las características antigénicas de la proteína VP6 se han descrito siete serogrupos o grupos antigénicos. Cada uno de estos grupos ha sido identificado por una letra de la A a la G. Los grupos A, B y C han sido aislados tanto en humanos como en animales, mientras que los grupos D, E, F y G han sido aislados únicamente de animales (Kapikian et al., 2001). No obstante, los rotavirus del grupo A son los causantes de la mayoría de las infecciones por rotavirus en humanos.

Los rotavirus del grupo A se asocian típicamente a diarrea en niños y animales jóvenes. El serogrupo B ha producido epidemias anuales de diarrea grave en adultos en China (Hung et al., 1983, Su et al., 1986, Wang et al., 1985) y en la India (Krishnan et al., 1999), y brotes de diarrea en recién nacidos en China (Mackow, 1995). Rotavirus pertenecientes al grupo C se han detectado en casos esporádicos de niños con diarrea (Jiang et al., 1995, Mackow, 1995, Otsu, 1998, Penaranda et al., 1989, Rodger et al., 1982) y en varios brotes (Caul et al., 1990, Hamano et al., 1999, Matsumoto et al., 1989).

Los rotavirus del grupo A se dividen en subgrupos (SG) dependiendo de la presencia o ausencia de dos epítopos reconocidos por los anticuerpos monoclonales 255/60 y 631/9 (Greenberg et al., 1983). De este modo nos encontramos cuatro SG diferentes: SG I; SG II; SG I+II y SG ni-I, ni-II. El SG II es el más frecuente entre las cepas humanas (Arista et al., 1990, Beards y Desselberger, 1989, Georges-Courbot et al., 1988, Iturriza-Gomara et al., 2001, Mohammed et al., 1994) mientras que el SG I es más frecuente entre las cepas de origen animal (Lopez et al., 1994, Tang et al., 1997).

Los rotavirus del grupo A también han sido caracterizados mediante ensayos de neutralización definiéndose así diferentes serotipos (Hoshino y Kapikian, 1996). Las proteínas implicadas en esta clasificación serológica son la glicoproteína VP7, que

define los serotipos G, y la hemaglutinina VP4, que define los serotipos P, ambas proteínas de la cápside externa de rotavirus. Así los rotavirus del grupo A se clasifican mediante un sistema binario que distingue los distintos serotipos de las proteínas VP7 y VP4 (Graham y Estes, 1985, Hoshino y Kapikian, 1996, Hoshino et al., 1984, Rodger y Holmes, 1979). Hasta el momento se conocen 15 serotipos G y 13 serotipos P (Estes, 2001). La denominación G y P son debidas a que la proteína VP7 es **g**licosilada (G) y a que la proteína VP4 es sensible a **p**roteasas (P). Debido a las dificultades intrínsecas de las técnicas serológicas utilizadas para la clasificación de los rotavirus del grupo A y la introducción de las técnicas moleculares, se estableció una nueva clasificación basada directamente en las secuencias de los genes VP7 y VP4. De este modo se distinguen 21 genotipos P en relación a la proteína VP4, de los cuales solo 16 se corresponden con serotipos conocidos de la proteína (Estes, 2001, Hoshino y Kapikian, 1996, Rao et al., 2000). Para la proteína VP7 existe una clara correlación entre genotipos y serotipos (Gouvea et al., 1990). La nomenclatura de los rotavirus del grupo A se ha consensuado de forma que en primer lugar se describe el serotipo G y posteriormente el serotipo P y/o el genotipo P entre corchetes. Como ejemplo, la cepa Wa sería G1 P1A[8].

4. Características estructurales de rotavirus

4.1. Estructura del virión

Los viriones de rotavirus están compuestos por tres capas proteicas que pueden ser observadas al microscopio electrónico (Figura 1). Las partículas infectivas completas, con un tamaño aproximado de 75 nm de diámetro, han sido históricamente llamadas partículas de doble capa, pero los datos estructurales actuales demuestran que poseen tres capas. La acción de productos quelantes del calcio, como el EDTA o el EGTA, desestabilizan las proteínas de la cápside externa (VP4 y VP7) pudiéndose

observar partículas de doble capa. Estas partículas (históricamente llamadas partículas de simple capa) poseen una morfología rugosa, cuando son observadas al microscopio electrónico, debido a la disposición trimérica de la proteína VP6. El tratamiento de los viriones de rotavirus con agentes caotrópicos tales como el cloruro cálcico permiten observar partículas de simple capa compuestas por las proteínas VP1, VP2 y VP3. Estas estructuras forman el core del virión y agregan con facilidad.

La estructura tridimensional de las partículas de doble y triple capa ha sido estudiada mediante técnicas de criomicroscopía y procesado de imágenes en partículas víricas no tripsinizadas (Prasad y Chiu, 1994, Prasad et al., 1988, Shaw et al., 1993, Yeager et al., 1994). Estos estudios muestran claramente que el número de triangulación (T) de rotavirus es 13, confirmando los resultados presentados por Roseto y colaboradores en 1979 (Roseto et al., 1979). Una característica importante de rotavirus es la presencia de 132 canales a lo largo de las dos cápsides externas que comunican el core de la partícula con el exterior.

Figura 1. Partículas de rotavirus observadas por microscopía electrónica tras tinción con 1% molibdato amónico, la barra representa 100 nm. El panel **a** muestra las partículas víricas completas, formadas por las proteínas estructurales VP1,VP2, VP3, VP4, VP6 y VP7. El panel **b** muestra las partículas de doble capa que carecen de las proteínas de cápside externa VP4 y VP7. El panel **c** muestra las partículas de simple capa o cores formadas por las proteínas VP1, VP2 y VP3 (Estes, 2001).

La función de estos canales no ha sido totalmente aclarada, aunque es probable que estén implicados en el transporte de los metabolitos necesarios para la transcripción del ARN viral y para la salida del ARN transcrito que será utilizado en los procesos posteriores de la replicación viral.

Estos estudios estructurales muestran la presencia de sesenta espículas (Prasad et al., 1988). Estas espículas están formadas por dímeros de la proteína VP4 y poseen una longitud aproximada de 120 Å. Estudios más recientes han confirmado que la longitud total de las espículas es de 200 Å y que éstas sobresalen 120 Å de la superficie viral (Shaw et al., 1993, Yeager et al., 1994). La proteína de la capa externa VP7 forma trímeros a partir de las 780 moléculas presentes en el virión. Esta proteína interacciona tanto con los trímeros de la proteína VP6 como con la proteína VP4.

Las partículas de doble capa tienen un radio de 720 Å aproximadamente y están compuestas por 780 moléculas de VP6 que definen un total de 260 unidades morfológicas formadas por trímeros de la proteína. Estudios realizados mediante la expresión de las diferentes proteínas que conforman el virión en el sistema de baculovirus demuestran que las proteínas estructurales de rotavirus poseen propiedades intrínsecas de auto-ensamblaje, permitiendo así la producción de partículas pseudovíricas (Crawford et al., 1994, Labbe et al., 1991).

4.2. Estructura del genoma

El genoma de rotavirus está compuesto por 11 segmentos de ARNbc que se encuentran localizados en el core del virión. El ARN viral necesita de interacciones ARN-proteína para adoptar la conformación necesaria y ser empaquetado en el virión (Kapahnke et al., 1986). Las proteínas implicadas en este proceso no han sido todavía descritas, aunque las proteínas estructurales presentes en el core del virión (VP1, VP2 y VP3) son claras candidatas. No obstante, proteínas no estructurales también pueden estar implicadas. Los fragmentos de ARN no son infectivos en ausencia de las proteínas virales, indicando que necesitan de la replicasa viral y que ésta se encuentra incluida en el virión.

Los once fragmentos del ARNbc viral poseen características y secuencias conservadas en todos los rotavirus secuenciados hasta el momento. Los segmentos

genómicos comienzan siempre con una guanina y están seguidos por secuencias conservadas en la zona no codificante. Tras la zona codificante de la proteína (ORF), al menos una por cada segmento genómico, también se encuentran secuencias conservadas (Figura 1.2). Los ORFs no se encuentran seguidos por secuencias de poliadenilación. Normalmente el codón de iniciación está precedido por una secuencia Kozak. Algunos de los genes poseen otros codones de iniciación en fase con la pauta de lectura a partir del primero (genes 7, 9 y 10) o fuera de fase (gen 11). Las evidencias de que se disponen indican que todos los genes son monocistrónicos excepto posiblemente los genes 9 y 11 (Chan et al., 1986, Mattion et al., 1991).

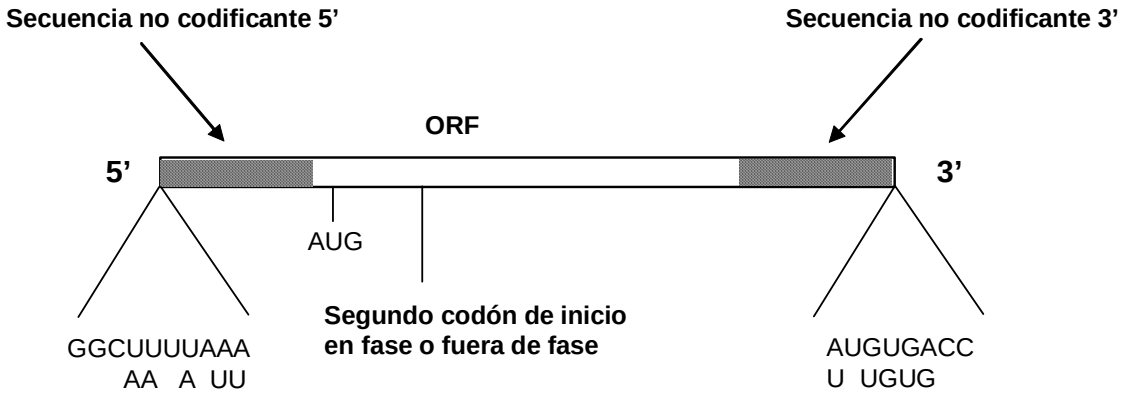

Figura 1.2. Representación esquemática de la estructura de un fragmento génico de rotavirus. En el esquema se muestran las secuencias consenso de las zonas no codificantes en ambos extremos del gen, así como la posible existencia de un segundo codón de inicio.

Los rotavirus son los únicos virus conocidos que infectan mamíferos y aves que poseen once segmentos de ARNbc. Esta característica ha permitido utilizar los patrones electroforéticos de las diferentes cepas de rotavirus para caracterizarlas, aunque el electroferotipo no puede ser utilizado como único método de clasificación debido a que cepas muy diferentes pueden compartir el mismo patrón (Estes, 2001). El número de cada segmento genómico de rotavirus ha sido asignado en referencia a su capacidad de migración en geles de poliacrilamida, siendo el número 1 el de menor movilidad electroforética y el 11 el de mayor. En los rotavirus de grupo A, el patrón

electroforético suele estar formado por 4 grupos de bandas (figura 1.3.). Cuatro segmentos (1-4) de gran peso molecular, dos de peso molecular intermedio (5-6), tres de pequeño tamaño (7-9) y dos de muy pequeño tamaño molecular (10 y 11). En algunas cepas aisladas en humanos el segmento que habitualmente codifica para las proteínas del segmento 11 migra más lentamente que el segmento décimo. Éste patrón es conocido como electroferotipo corto y casi siempre corresponde a cepas del subgrupo antigénico I de rotavirus humanos (Hoshino y Kapikian, 1996). El electroferotipo largo es característico de rotavirus humanos pertenecientes al subgrupo II.

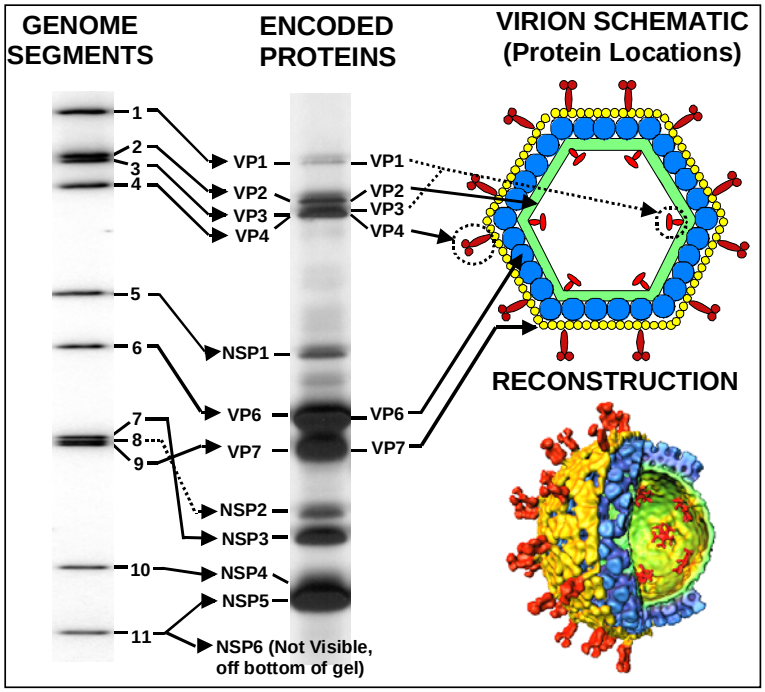

Figura 1.3. Asignación de las proteínas codificadas por el genoma de rotavirus. La imagen muestra el fragmento genético que codifica para cada una de las proteínas y la ubicación en el virión de las proteínas estructurales de rotavirus. (Figura reproducida de E. Mossel, M. Estes y F. Ramig, http://www.iah.bbsrc.ac.uk).

Los genes de rotavirus pueden sufrir reordenamientos genómicos, en estos casos los patrones electroforéticos presentan anomalías, de modo que aparecen bandas que migran más lentamente y las bandas correspondientes a los segmentos implicados desaparecen o se observa un brusco descenso de su concentración

(Hundley et al., 1985). Generalmente las cepas víricas que presentan reordenamientos en su genoma no son defectivas y los concatémeros resultantes del reordenamiento sustituyen funcionalmente a los segmentos implicados (Allen y Desselberger, 1985, Biryahwaho et al., 1987, Graham et al., 1987).

4.3. Estructura y función de las proteínas de rotavirus

Los once fragmentos de rotavirus codifican para un total de 12 proteínas. La asignación de cada una de estas proteínas al fragmento correspondiente de ARNbc se ha realizado mediante experimentos de traducción *in vitro* (Dyall-Smith y Holmes, 1981b, Mason et al., 1980, Mason et al., 1983, McCrae y Faulkner-Valle, 1981, Smith et al., 1980). La figura 1.3 muestra la relación entre cada segmento de ARNbc, la proteína que codifica y la posición que cada una de ellas ocupa dentro de la estructura del virión.

Las principales características de las proteínas de rotavirus se encuentran resumidas en la tabla 1.1.

Algunas de las proteínas de rotavirus han sido ampliamente estudiadas debido a su importancia en el ciclo replicativo de rotavirus, sus características antigénicas o su relevancia en la patogenia vírica.

4.3.1. Proteína VP4

La proteína VP4 es el producto del cuarto segmento genómico, tiene un peso molecular de 88 KD y dimeriza formando las espículas de la cubierta externa del virión. Esta proteína posee una longitud de 776 aminoácidos, o 775 en el caso de la mayoría de las cepas aisladas en humanos, en las cuales falta el aminoácido situado entre las posiciones 134 y 136. Esta proteína es un importante determinante antigénico, ya que es capaz de inducir la producción de anticuerpos neutralizantes de la infección por

rotavirus (Hoshino et al., 1995). Esta característica de la proteína VP4 permite la clasificación de los rotavirus del grupo A en diferentes serotipos P. También es una proteína clave en la infectividad (Greenberg et al., 1983, Offit et al., 1986). VP4 posee la capacidad de aglutinar hematíes, actuando como una hemaglutinina viral. Esta capacidad hemaglutinante no se encuentra presente en las cepas de rotavirus humanos.

Tabla 1.1. Proteínas codificadas por el genoma de rotavirus

Segmento Genómico	Producto proteico	Localización	Características y función
1	VP1	core	ARN polimerasa, unión a ARN, unión a VP3
2	VP2	core	Unión a ARN bc, requerida para la actividad replicasa de VP1
3	VP3	core	Guanilil transferasa, metil transferasa, unión a ARNmc, unión a VP1
4	VP4	cápside externa	Dimérica, hemaglutinina, antígeno neutralizante, procesada por proteasas, espícula, adhesión celular, serotipos P
5	NSP1	no estructural	Dedos de zinc, unión a ARNsc, unión al citoesqueleto
6	VP6	cápside interna	Hidrofóbica, trimérica, antígeno de subgrupo
7	NSP3	no estructural	Ácida, unión a ARNm viral, interacciona con elF4G1, inhibe traducción del hospedador
8	NSP2	no estructural	Básica, oligomérica, unión a ARNsc, NTPasa
9	VP7	cápside externa	Glicoproteína, trimérica, antígeno neutralizante, unión a Calcio, serotipos G
10	NSP4	no estructural	Glicoproteína, receptor intracelular en el retículo endoplasmático, morfogénesis, enterotoxina
11	NSP5	no estructural	Básica, fosfoproteína, unión ARNsc, proteína quinasa, interacción con VP2, NSP2, NSP6
	NSP6	no estructural	Interacción con NSP5, se localiza en el viroplasma

La proteína VP4 de rotavirus es escindida en dos polipéptidos por la acción de la tripsina, esta escisión ocurre en aminoácidos altamente conservados (figura 1.4), éstos son la arginina 241 y la arginina 247, aunque la arginina 247 es el lugar de escisión mayoritario (Lopez et al., 1985). La acción de proteasas sobre la proteína VP4

potencia la infectividad de rotavirus (Espejo et al., 1981, Estes et al., 1981, Ramia y Sattar, 1980, Wyatt et al., 1983). Mediante criomicroscopía electrónica se han demostrado cambios conformacionales en las espículas de rotavirus cuando son incubados con concentraciones crecientes de tripsina (Crawford et al., 2001), aunque los cambios estructurales y fisicoquímicos ocurridos en las espículas después de la proteolisis no están claramente definidos. Los productos de la proteolisis de esta proteína son las proteínas VP5* y VP8*. La proteína VP8*, de 28 KD, comprende el fragmento amino terminal de la proteína VP4 mientras que la proteína VP5* de 60 KD queda contenida en el extremo carboxi terminal de la proteína VP4.

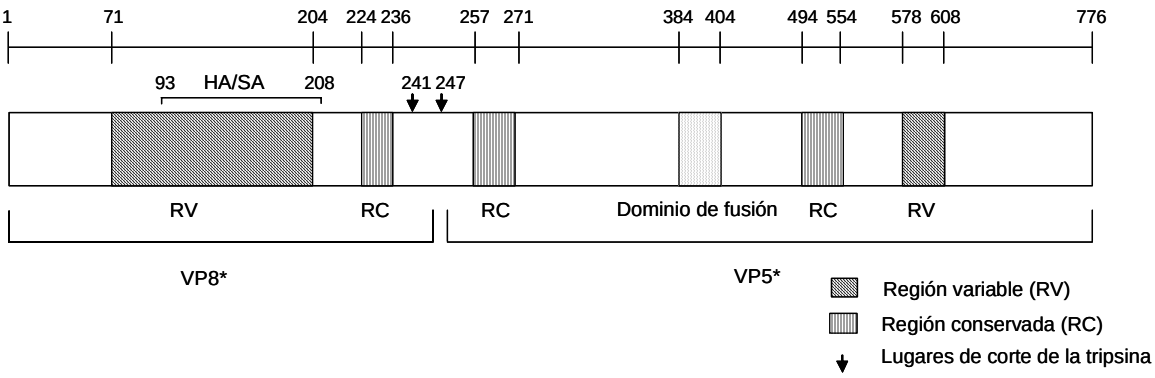

Figura 1.4. Representación esquemática de la proteína de VP4 de rotavirus basada en diferentes cepas de rotavirus. Se muestra el dominio de aglutinación y unión a ácido siálico (HA/SA), las zonas de corte de la tripsina y las regiones conservadas y variables de la proteína.

Experimentos realizados con VP8* producida tanto en *E. coli* (Lizano et al., 1991) como en células de insecto (Fiore et al., 1991) muestran que la capacidad hemaglutinante de la proteína VP4 reside dentro de la porción que da lugar a la proteína VP8*. Estudios posteriores confirmaron que esta región debe encontrarse entre los aminoácidos 93 y 207 de la proteína VP8* (Fuentes-Panana et al., 1995). Dentro de la proteína VP5*, entre los aminoácidos 384 y 401 de VP4, se encuentra una región altamente conservada dentro de todas las cepas de rotavirus. Esta región comparte una alta homología con los lugares de fusión que se encuentran en los

receptores de otros virus tales como los alphavirus Semliki Forest y el virus Sindbis (Mackow et al., 1988). De forma que esta zona de la proteína VP5* posiblemente esté implicada en el proceso de entrada del virus en la célula hospedadora durante el proceso de internalización del virus.

4.3.2. Proteína VP6

La proteína VP6 es una proteína altamente hidrofóbica e inmunogénica con un peso molecular de 45 KD y es el principal componente estructural de los viriones. Esta proteína de la capa intermedia de rotavirus interacciona al mismo tiempo con la proteína VP2 de la cápside interna y con las proteínas VP4 y VP7 de la cápside externa de rotavirus (Estes, 2001). La proteína VP6 forma trímeros espontáneamente y es extremadamente estable (Estes et al., 1987). Estas características junto con la presencia de epítopos altamente conservados entre diferentes cepas víricas hacen que sea el antígeno utilizado en la mayoría de las técnicas de diagnóstico de infecciones por rotavirus (Estes y Cohen, 1989). La proteína VP6 también interacciona con la proteína NSP4 de rotavirus. Esta interacción es la que dirige a las partículas víricas inmaduras hacía el interior del retículo endoplasmático (Meyer et al., 1989). Esta proteína también ha sido relacionada con la maquinaria replicativa de rotavirus. La adición de agentes caotrópicos a partículas de doble capa disocia las moléculas de VP6 (figura 1.1) dejando al descubierto los cores víricos. Estas partículas de simple capa son incapaces de replicar el ARN viral pero en cuanto las condiciones del medio permiten la reasociación de VP6 a los cores, éstos recuperan la actividad ARN polimerasa (Bican et al., 1982, Estes y Cohen, 1989, Sandino et al., 1986).

Se han determinado los dominios implicados en las diferentes funciones y características estructurales de esta proteína mediante análisis realizados con diferentes variantes virales y proteínas quiméricas (figura 1.5). El dominio de trimerización de la proteína se encuentra entre los aminoácidos 246 y 314 y la fracción

necesaria para la formación de las partículas de doble capa ha sido mapeada en los residuos 353 al 397 (Affranchino y Gonzalez, 1997, Tosser et al., 1994). Los aminoácidos 296-299 y 305 determinan el epítopo del subgrupo I reconocido por el anticuerpo monoclonal 255/60 mientras que el residuo 315 es uno de los implicados en la especificidad del subgrupo II (Lopez et al., 1994, Tang et al., 1997).

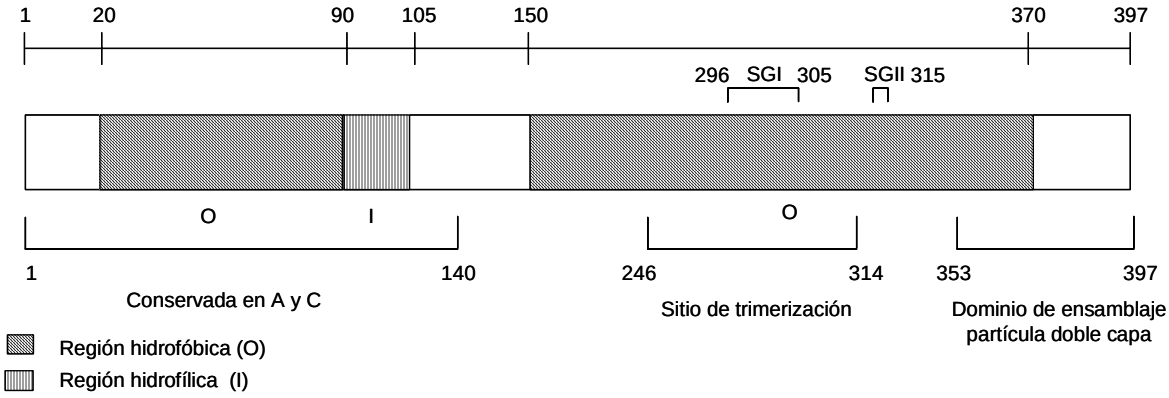

Figura 1.5. Representación esquemática de la proteína de VP6 de rotavirus Se muestran los dominios hidrofóbicos e hidrofílicos de la proteína, así como los epítopos responsables de la división en subgrúpos y las regiones conservadas y variables de la proteína.

4.3.3. Proteína VP7

La proteína VP7 está codificada por el segmento 9 de rotavirus en la mayoría de las cepas estudiadas, aunque la movilidad electroforética de este segmento puede variar en determinadas cepas, migrando en posición 7 en la cepa RRV o el octavo lugar en la cepa UK (Dyall-Smith y Holmes, 1981a).

VP7 es una proteína glicosilada que forma parte de la cápside externa del virión de rotavirus. La secuencia de glicosilación puede variar de unas cepas a otras, siendo la secuencia Asn-Ser-Thr, en posiciones 69-71, el lugar de glicosilación habitual (Gunn et al., 1985, Kouvelos et al., 1984, Nishikawa et al., 1989).

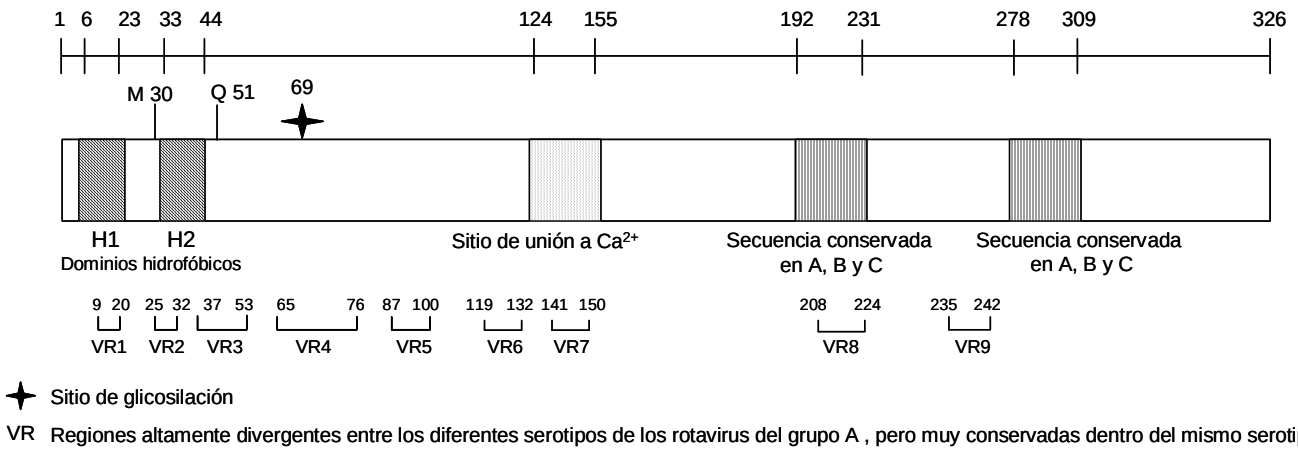

Figura 1.6. Representación esquemática de la proteína de VP7 de rotavirus. Se muestran los dominios hidrofóbicos de la proteína, así como los epítopos responsables de la división en serotipos. El segundo codón de iniciación está indicado como M 30 y el sitio de corte del péptido señal como Q 51.

El gen de la proteína VP7 codifica para 326 aminoácidos (figura 1.6). El ORF comienza con un codón de iniciación precedido por una secuencia de iniciación débil, seguido por una zona hidrofóbica y un segundo codón de iniciación esta vez precedido por una secuencia consenso fuerte. Este segundo codón de iniciación también está seguido por una zona hidrofóbica (figura 1.6). Estas secuencias hidrofóbicas deben ser las encargadas de dirigir a la proteína VP7 al retículo endoplasmático, donde la glicosilación es llevada a cabo durante la entrada de la proteína. El péptido señal que dirige a la proteína VP7 al retículo es escindido en la glicina 51, en la mayoría de las cepas de rotavirus, de forma que los dos dominios hidrofóbicos no están presentes en las proteínas maduras (Stirzaker et al., 1987). Mediante estudios realizados con enzimas proteolíticos se ha demostrado que la proteína VP7 queda insertada en el retículo endoplasmático orientada hacía el lumen, debido a que una vez asociada a membranas es resistente a la digestión por estos enzimas.

Las proteínas glicosiladas de rotavirus (VP7 y NSP4) quedan retenidas en el retículo endoplasmático y no continúan la ruta de secreción seguida por la mayoría de proteínas glicosiladas. En el caso de la proteína VP7 se han identificado dos regiones que podrían estar implicadas en esta retención (aminoácidos 51-61 y 61-111), aunque

el mecanismo no está totalmente aclarado (Poruchynsky y Atkinson, 1988). Estudios más recientes muestran como la proteína NSP4 es capaz de impedir que las vesículas nacientes del retículo endoplasmático sigan su ruta hacia el aparato de Golgi (Xu et al., 2000) pudiendo este mecanismo explicar por qué estas proteínas glicosiladas no siguen la ruta de secreción habitual.

Para la maduración de las partículas víricas es imprescindible la presencia de Ca^{2+}. Si el calcio es eliminado de las células infectadas mediante ionóforos o si es inhibido mediante un competidor como el Mn^{2+}, la maduración de las partículas víricas es abortada (Poruchynsky et al., 1991) La proteína VP7 forma hetero-oligómeros junto con NSP4 y VP4 en el retículo endoplasmático. Éstos son estabilizados mediante calcio que se une a la proteína VP7. Así mismo, si el calcio es eliminado mediante quelantes, tales como EGTA o EDTA, de las partículas víricas maduras, éstas pierden su envoltura externa.

VP7 también ha sido utilizada como antígeno para definir los serotipos G de rotavirus, atendiendo a la presencia de anticuerpos neutralizantes que reconocen esta proteína.

4.3.4. Proteína NSP4

La proteína NSP4 de rotavirus se encuentra codificada por el segmento 10 del genoma viral. El estudio sobre esta glicoproteína no estructural se ha intensificado en los últimos años de forma notable debido a su importancia tanto en la morfogénesis como en la fisiopatología de rotavirus.

La secuencia de la proteína NSP4 procedente de los rotavirus del gupo A consta de 175 aminoácidos (figura 1.7.); en la porción amino-terminal de la proteína se encuentran tres zonas hidrofóbicas (Estes, 2001) que son las encargadas de dirigir la proteína hacia el retículo endoplasmático donde la proteína será glicosilada. La

porción carboxi-terminal es hidrofílica y queda expuesta hacía el citoplasma celular. En esta región se encuentran la mayoría de las actividades de la proteína NSP4.

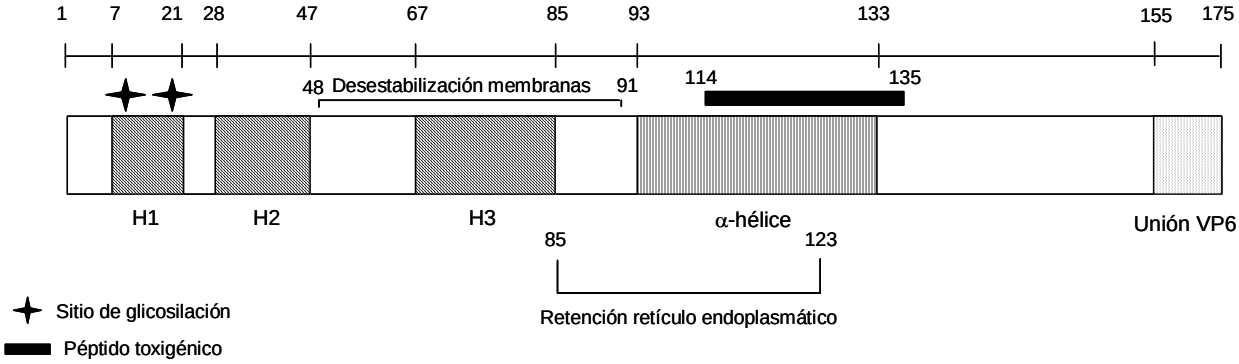

Figura 1.7. Representación esquemática de la proteína NSP4 de rotavirus. Se muestran los dominios hidrofóbicos de la proteína, el péptido toxigénico y el sitio de unión a VP6. Los dos sitios de glicosilación se encuentran en la primera región hidrofóbica (H1) que es la única que queda orientada hacia el lumen.

La proteína NSP4 actúa como receptor intracelular en el proceso de morfogénesis de rotavirus, mediante el cual las partículas víricas inmaduras son internalizadas en el retículo endoplasmático. La región de la proteína implicada en este reconocimiento se encuentra en los últimos 20 aminoácidos localizados en la cola citoplasmática de la proteína. (Au et al., 1989, Au et al., 1993, Meyer et al., 1989, O'Brien et al., 2000, Olivo y Streckert, 1995, Taylor et al., 1993, Taylor et al., 1996). La proteína NSP4, al igual que la proteína VP7, queda retenida en el retículo endoplasmático. No obstante, ninguna de estas proteínas de rotavirus posee secuencias típicas de retención en el retículo, de forma que deben utilizar un mecanismo diferente a los descritos hasta ahora para ello. Estudios recientes muestran que la proteína NSP4 bloquea el tráfico de membranas del retículo endoplasmático hacia el aparato de Golgi (Xu et al., 2000) y la región implicada en la retención de NSP4 en el Golgi está comprendida entre los aminoácidos 85 y 123 de la región citoplasmática de NSP4 (Mirazimi et al., 2003). Otra de las funciones que han sido otorgadas a la proteína NSP4 de rotavirus durante la morfogénesis viral es su capacidad para desestabilizar membranas. Esta actividad estaría implicada en la pérdida de la envuelta lipídica que poseen los rotavirus después de su internalización

en el retículo endoplasmático. Esta capacidad se encuentra entre los aminoácidos 48 a 91 de la proteína (Browne et al., 2000, Tian et al., 1996).

Una de las características de la proteína NSP4 es su papel en la patogénesis viral. En 1996 Ball y colaboradores (Ball et al., 1996) mientras realizaban experimentos de inmunización en ratones lactantes descubrieron que tanto la proteína completa como el péptido sintético que codifica los aminoácidos del 114 al 135 de la proteína NSP4 ($NSP4_{114-135}$) eran capaces de producir diarrea. De este modo la proteína NSP4 pasó a ser la primera enterotoxina de origen vírico que se describía. El proceso por el cual la proteína NSP4 o el péptido $NSP4_{114-135}$ actúan como toxina está asociado a su capacidad de movilizar calcio del retículo endoplasmático hacia el citoplasma, estimulando así la salida de iones cloruro y agua al exterior celular (Ball et al., 1996, Dong et al., 1997, Morris et al., 1999, Tian et al., 1995, Tian et al., 1994).

5. Ciclo replicativo de rotavirus

La replicación de rotavirus ha sido estudiada principalmente en cultivos de células MA104. En esta línea celular el ciclo replicativo de rotavirus es relativamente rápido, de forma que se observa un máximo de producción vírica de 10 a 12 horas post-infección si las células son cultivadas a 37º C, o a las 18 horas si el cultivo celular se realiza a 33º C. Las características generales del ciclo replicativo de rotavirus en células MA104 se pueden resumir en los siguientes puntos:

1. El cultivo de la mayoría de las cepas de rotavirus requiere la adición de proteasas al medio de cultivo. Las proteasas digieren la proteína VP4 de la cápside externa de rotavirus activando la infectividad del virus.
2. La replicación es totalmente citoplasmática.
3. Las células infectadas no poseen ningún enzima capaz de replicar el ARNbc que forma el genoma de rotavirus, de forma que el virus debe proporcionar los enzimas necesarios para la replicación.

4. El ARN transcrito es utilizado tanto para la producción de las proteínas virales tras su traducción en los ribosomas, como para la producción de la hebra negativa de ARN que formará el ARNbc. Una vez la hebra negativa de ARN es sintetizada se mantiene unida a la hebra positiva.
5. Los segmentos de ARNbc se sintetizan en el interior de las partículas subvirales. Tanto el ARNbc como los segmentos de ARN negativo no se encuentran libres en el citoplasma de las células infectadas.
6. Las partículas subvirales maduran mediante su internalización en el retículo endoplasmático. Durante este proceso las partículas adquieren las proteínas de la cápside externa.
7. Las partículas víricas son liberadas tras la lisis celular.

5.1 Adsorción, penetración y decapsidación

El proceso de unión de rotavirus a la superficie celular es mediado por múltiples interacciones que varían según la cepa de rotavirus estudiada (Mendez et al., 1999). La figura 1.7. muestra un resumen de estas interacciones. Para un gran número de cepas aisladas de animales, una primera etapa de la adsorción vírica es mediada por la unión de la porción VP8* de la proteína VP4 a ácido siálico (Mackow et al., 1989). La mayoría de las cepas de rotavirus aisladas en humanos no requieren de la interacción con ácido siálico (Ciarlet y Estes, 1999); en estas cepas la unión al ganglíosido GM1 parece ser la implicada en esta primera etapa de la adsorción (Guo et al., 1999). En una segunda etapa de la adsorción para las cepas dependientes de unión a ácido siálico y en una primera o segunda etapa para las cepas que no dependen de esta unión, se han identificado interacciones proteína-proteína. La integrina $\alpha 2\beta 1$ interaccionaría con la proteína VP4, mientras que la integrina $\alpha 4\beta 1$ interaccionaría con la proteína VP7 (Coulson et al., 1997, Hewish et al., 2000). La secuencia de unión de VP4 a la integrina $\alpha 2\beta 1$ ha sido mapeada en el tripéptido de

secuencia DGE que se encuentra entre los aminoácidos 308 y 310 de la proteína VP5* (Zarate et al., 2000). Secuencias peptídicas de unión a integrinas, tales como LDV, LDI y IDI, están presentes en la proteína VP7 de todas las cepas de rotavirus aisladas de mamíferos (Coulson et al., 1997). Una de las integrinas implicadas en el proceso de adsorción y entrada de rotavirus en las células infectadas es la integrina $\alpha V\beta 3$. El motivo de unión a esta integrina se encuentra en la proteína VP4 y estudios con anticuerpos monoclonales frente a esta integrina son capaces de bloquear la infectividad de rotavirus, aunque no su adsorción (Guerrero et al., 2000a). La chaperona hsc70, que forma parte de la familia de las chaperonas hsp70, también se encuentra implicada en el proceso de adsorción e internalización vírica. La entrada de rotavirus al interior celular puede ser inhibida incubando los rotavirus con esta chaperona, de forma que se bloquea la unión de las proteínas VP4 y VP7 a este receptor celular. Este bloqueo realizado con la proteína hsc70 inhibe la entrada del virus al interior celular, pero no la unión del virus a las células (Guerrero et al., 2002).

Estudios bioquímicos que muestran la participación de glicoproteínas, glicolípidos, colesterol y otras proteínas e integrinas relacionadas con la adsorción y entrada del virus forman parte de microdominios lipídicos (“rafts”) en la membrana celular. Se ha propuesto que son estas estructuras las que actuarían como receptores de rotavirus en las células, y no una única proteína (Cuadras y Greenberg, 2003, Guerrero et al., 2000b).

La entrada de rotavirus al interior celular es un proceso sobre el que todavía no existe un claro consenso, ya que la entrada puede ocurrir tanto por endocitosis como por entrada directa del virus. Tanto la proteína VP4 (Ruiz et al., 1997) como la proteína VP7 (Charpilienne et al., 1997) poseen la capacidad de permeabilizar membranas, de forma que estas proteínas parecen estar directamente implicadas en la entrada del virus en la célula. En los primeros estudios realizados sobre la entrada de rotavirus en las células hospedadoras se describió que tanto la cepa SA11 (Petrie et al., 1983,

Quan y Doane, 1983) como la cepa OSU (Ludert et al., 1987) eran internalizados en las células mediante endocitosis. Una vez en el interior celular las partículas víricas son parcialmente decapsidadas debido a las bajas concentraciones de calcio intracelular, ya que el uso de ionóforos que aumentan la concentración de calcio intracelular bloquean el proceso de decapsidación (Ludert et al., 1987).

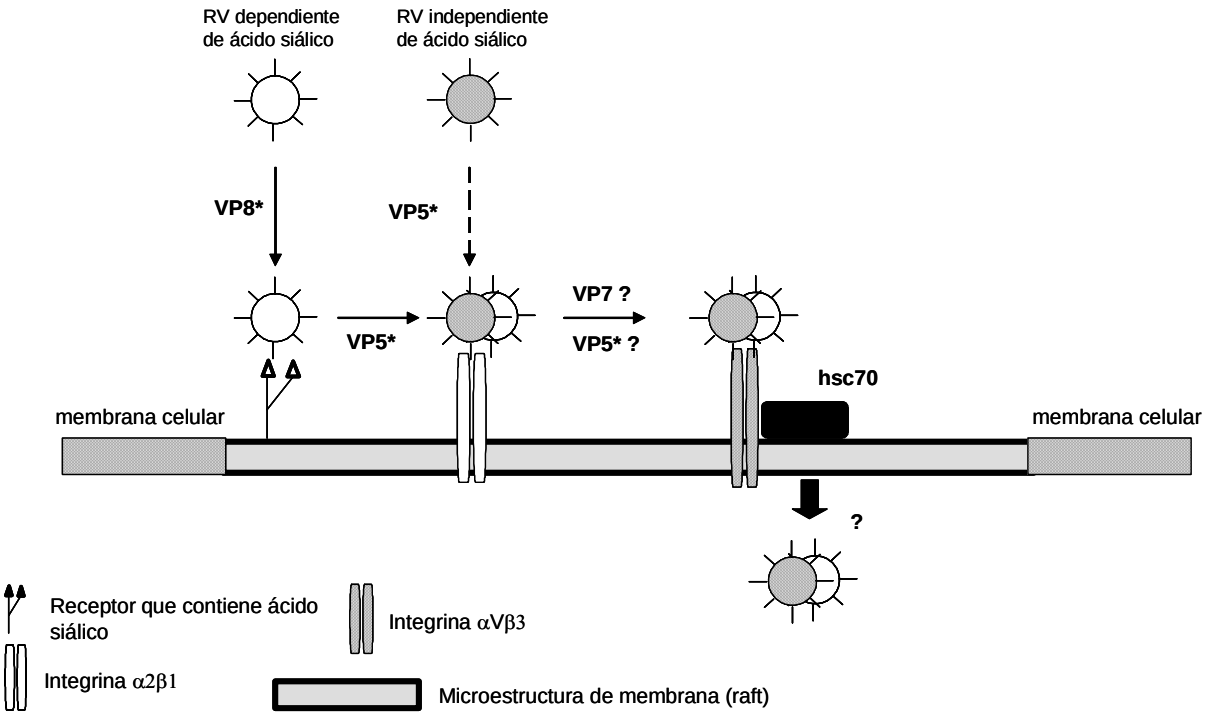

Figura 1.7. Representación esquemática de la adsorción y entrada de rotavirus en la célula infectada. Las cepas dependientes de ácido siálico interaccionan en un primer paso con un receptor de membrana que contiene ácido siálico mediante un dominio localizado en la proteína VP8* y posteriormente con la integrina $\alpha 2\beta 1$. Las cepas de rotavirus independientes de ácido siálico interaccionan directamente con la integrina $\alpha 2\beta 1$ o previamente con el ganglíosido GM1. Como muestra la imagen la integrina $\alpha V\beta 3$ y la chaperona hsc70 también están implicadas en este proceso.

Estudios realizados con virus humanos tripsinizados y sin tripsinizar muestran que las partículas activadas mediante proteasas atraviesan la membrana celular de forma directa, mientras que las partículas no tratadas son fagocitadas. Como resultado de las dos diferentes vías de entrada de rotavirus, las partículas que fueron

fagocitadas no fueron capaces de replicar (Suzuki et al., 1985, Suzuki et al., 1986). La mayoría de los anticuerpos monoclonales con actividad neutralizante que reconocen la proteína VP4 reconocen la porción VP5* de la proteína, sugiriendo que la entrada de los rotavirus al interioir celular está mediada por la proteína VP5* (Kirkwood et al., 1996, Kobayashi et al., 1990, Padilla-Noriega et al., 1995). Estudios recientes muestran que existen al menos dos dominios en la proteína VP5* necesarios para la formación del poro por donde entran los rotavirus de forma directa. Un dominio básico en la región amino terminal de la proteína que interacciona superficialmente con la membrana celular y un dominio hidrofóbico interno esencial para alterar la permeabilidad de la membrana (Golantsova et al., 2004).

Una vez las partículas víricas se encuentran en el citoplasma celular las proteínas de la cápside externa se pierden debido a la baja concentración de calcio, quedando de este modo libre las partículas parcialmente decapsidadas (VP1, VP2, VP3, VP6) con capacidad replicativa.

5.2. Transcripción y replicación

La síntesis de los transcritos virales está mediada por el complejo transcripcional de rotavirus, ya que las células eucariotas no poseen ningún enzima capaz de sintetizar ARN a partir de un molde de ARN (ARN polimerasa ARN dependiente). Los primeros estudios realizados tanto “in vitro” (Cohen y Dobos, 1979, Spencer y Arias, 1981) como “in vivo” (Bass et al., 1992) muestran que la actividad transcriptasa de rotavirus se encuentra en las partículas de doble capa. Una vez las partículas de doble capa (VP1, VP2, VP3, VP6) se encuentran en el citoplasma celular comienza el proceso de transcripción y replicación. Las actividades enzimáticas de la replicasa de rotavirus se han descrito mediante estudios de transcripción “in vitro”. Las partículas de rotavirus poseen actividad transcriptrasa, nucleótido fosfohidrolasa,

guanililtransferasa, metilasa y poli(A)-polimerasa (Cohen, 1977, Mason et al., 1980, McCrae y McCorquodale, 1982, Sandino et al., 1986).

La transcripción y replicación de rotavirus ocurre en unas estructuras subcelulares localizadas en el citoplasma de las células llamadas viroplasmas. Estas estructuras están formadas por las proteínas estructurales VP1, VP2, VP3 y VP6, por ARN vírico y por las proteínas estructurales NSP2 y NSP5 principalmente.

La transcripción en rotavirus es un proceso semiconservativo y todos los transcritos están compuestos por una hebra de ARN positivo completa sintetizada a partir de la hebra negativa del ARNbc (McCrae y McCorquodale, 1983). Los ARN mensajeros nacientes salen al exterior de las partículas de doble capa por los poros existentes en los ejes de simetría quíntuple de las partículas subvíricas. Los mensajeros poseen la caperuza, que es incorporada por VP3 en 5', pero carecen de colas de poliadenina en su extremo 3' (Estes, 2001, Jayaram et al., 2004, Taraporewala y Patton, 2004). Los transcritos de rotavirus, a pesar de carecer de las colas de poliadenina, son estabilizados en su extremo 3' mediante la unión de la proteína no estructural NSP3 a una secuencia de tan sólo cuatro nucleótidos (Poncet et al., 1993, Poncet et al., 1994). Otras proteínas no estructurales de rotavirus tales como NSP2 y NSP5 son parte importante del complejo de polimerización del ARN viral (Taraporewala y Patton, 2004).

La replicación del ARN vírico ocurre en la misma maquinaria replicativa que la transcripción, pero en este caso el ARNbc se forma a partir de la hebra positiva de ARN. La síntesis del ARNbc y el empaquetado del mismo en los nuevos viriones ocurren de forma prácticamente simultánea (Patton y Gallegos, 1988, Patton y Gallegos, 1990), de forma que no se ha aislado ARN negativo o de doble cadena de los citoplasmas de las células infectadas.

5.3. Morfogénesis y liberación de las partículas víricas

El proceso de morfogénesis de rotavirus se caracteriza por la internalización de las partículas inmaduras en el retículo endoplasmático y por encontrarse envueltas transitoriamente.

Las partículas subvirales, que ya contienen el material genético y las proteínas del core (VP1, VP2 y VP3) y de la cápside intermedia (VP6), son dirigidas desde los viroplasmas al interior del retículo endoplasmático. Este proceso está mediado por la interacción de la proteína VP6 con la glicoproteína no estructural NSP4, que actúa como receptor de las partículas subvirales en el retículo endoplasmático (Au et al., 1989, Au et al., 1993, Meyer et al., 1989, O'Brien, 2000, Olivo y Streckert, 1995, Taylor et al., 1993, Taylor et al., 1996). Durante la entrada de las partículas al interior del retículo endoplasmático, éstas adquieren una envuelta lipídica que desaparece durante los siguientes procesos de la morfogénesis viral. La alta concentración de calcio presente en el interior del retículo endoplasmático es importante para el proceso de morfogénesis. Si el calcio es eliminado del interior del retículo el proceso de morfogénesis queda bloqueado en el punto en el que las partículas subvirales se encuentran envueltas (Michelangeli et al., 1995, Ruiz et al., 2000). El proceso de maduración requiere de la desaparición de la envuelta lipídica y del ensamblaje de las proteínas estructurales VP7 y VP4 que conforman la cápside externa. Según el modelo propuesto por Tian y colaboradores en 1996 (Tian et al., 1996) sería la proteína NSP4 la que actuaría desestabilizando la bicapa lipídica de las partículas envueltas. Esta teoría se encuentra apoyada por diferentes estudios en los que se demuestra que la proteína NSP4 posee capacidad de interaccionar con bicapas lipídicas y desestabilizarlas (Browne et al., 2000, Huang et al., 2004, Huang et al., 2001, Newton, 1997). La proteína NSP4 también actúa como receptor de glicoproteína VP7 y la proteína VP4 con las que forma hetero-oligómeros. Finalmente las proteínas VP4 y VP7 se ensamblan en las partículas víricas nacientes del retículo

endoplasmático formando las partículas víricas completas. En este proceso la presencia de calcio también resulta importante ya que si el calcio es eliminado de las células infectadas mediante ionóforos o si es inhibido mediante un competidor como el Mn^{2+}, la maduración de las partículas víricas es abortada (Poruchynsky et al., 1991).

La liberación de rotavirus de las células infectadas puede ocurrir antes de la lisis celular si la infección ocurre en células epiteliales polarizadas, tales como las células Caco-2, en las cuales los virus son secretados por la zona apical de las células y son transportadas hasta allí mediante un mecanismo de transporte no convencional en el que el aparato de Golgi no participa (Jourdan et al., 1997). En células no polarizadas tales como la línea celular MA104 las partículas víricas son liberadas al exterior celular tras la lisis celular.

Estudios recientes muestran que los rotavirus utilizan las microestructuras de membrana lipídica ("rafts") durante su replicación para ser transportadas hasta la superficie celular tanto en experimentos realizados "in vitro" como "in vivo". Según esta teoría los rotavirus utilizarían las mismas rutas de transporte que utilizan los diferentes componentes de estas microestructuras de membrana en las que se forman zonas diferenciadas de la membrana celular, explicando de este modo como los rotavirus son excretados al exterior celular por la zona apical de las células epiteliales polarizadas (Cuadras y Greenberg, 2003, Sapin et al., 2002).

6. Fisiopatología de la infección por rotavirus y la proteína NSP4

Se han sido realizados numerosos esfuerzos para determinar los mecanismos involucrados en la patogénesis de las infecciones por rotavirus (Estes et al., 2001). La diarrea inducida por rotavirus se atribuye a tres mecanismos diferenciados, que deben contribuir a la diarrea producida por rotavirus en diferentes momentos de la infección vírica (Morris y Estes, 2001):

1) Una reducción en la superficie de absorción del intestino delgado, que determina una disminución de la capacidad absortiva de agua.

2) Cambios en la permeabilidad osmótica de la mucosa intestinal.

3) Cambios en la secreción de fluidos y electrolitos.

El incremento de calcio que se produce tras la infección por rotavirus provoca citolisis en células epiteliales no polarizadas (Estes, 2001). Por otra parte la infección por rotavirus o la adición de la proteína NSP4 produce la pérdida de la integridad del epitelio en células polarizadas (Tafazoli et al., 2001). Estas actividades deben jugar un papel importante en la destrucción de los *villi* del intestino que se observa en la infección por rotavirus *in vivo*. La lesión intestinal también puede estar asociada con una respuesta inmunopatológica. Este mecanismo para la producción de diarrea se relaciona con un gran componente inflamatorio que no se observa ni en humanos ni en el modelo de infección murino (Casola et al., 1998, Estes y Morris, 1999, Franco y Greenberg, 2000). La implicación de la proteína NSP4 en la diarrea inducida por rotavirus fue postulado por primera vez después de la expresión de las diferentes proteínas de rotavirus en cultivos celulares. La proteína NSP4 fue la única capaz de inducir un incremento en la concentración de calcio intracelular mimetizando la infección por rotavirus (Dong et al., 1997, Tian et al., 1995, Tian et al., 1994). Este calcio es movilizado desde el retículo endoplasmático al citoplasma celular por medio de la activación de la fosfolipasa C cuando la proteína es añadida extracelularmente a

las células (Dong et al., 1997, Tian et al., 1995). También se ha propuesto la hipótesis de que la proteína NSP4 formaría canales en el retículo endoplasmático que mediarían la salida de calcio al citoplasma cuando la proteína es producida intracelularmente (Morris y Estes, 2001, Tian et al., 1995, Tian et al., 1994). La importancia de la proteína NSP4 en la fisiopatología de rotavirus fue confirmada durante experimentos de inmunización en ratones con la proteína NSP4, que demostró actuar como una enterotoxina produciendo diarrea en ratones recién nacidos. Esta actividad toxigénica también fue producida por el péptido sintético conteniendo los aminoácidos 114 a 135 ($NSP4_{114-135}$) de la proteína (Ball et al., 1996) y confirmada en estudios posteriores (Horie et al., 1999, Mori et al., 2002a, Morris et al., 1999, Rodríguez-Díaz et al., 2003).

Otro de los factores implicados en la diarrea inducida por rotavirus es la participación del sistema nervioso entérico. Según los resultados publicados por Lundgren y colaboradores (Lundgren et al., 2000) más del 60% de la secreción neta de fluidos durante la diarrea por rotavirus es mediada por el arco reflejo secreto-motor. Pero hasta el momento no se conoce el nexo entre la infección por rotavirus y la activación del sistema nervioso entérico. Se ha propuesto que la isquemia producida por rotavirus puede provocar cambios significativos en la secreción de óxido nítrico en las células afectadas y que esta molécula actuaría como neurotransmisor activando el sistema nervioso entérico, aunque este aspecto no ha sido todavía demostrado (Morris y Estes, 2001). El papel que puede jugar la proteína NSP4 en esta activación no ha sido investigado todavía, pese a que otras enterotoxinas tales como las toxina termosensible y termoestable A de *E. coli*, así como la toxina colérica, ejercen su capacidad enterotoxigénica después de la activación de las rutas secreto-motoras del sistema nervioso entérico (Farthing, 2000).

6.1. Modelo de la inducción de la diarrea por rotavirus

La producción de la diarrea por parte de rotavirus es un proceso complejo en el que se encuentran implicados tanto la propia infección vírica como la respuesta del

hospedador. La mayoría de los datos que se dispone hasta el momento proviene de experimentos realizados tanto en animales como en diferentes líneas celulares, aunque algunos de ellos han sido confirmados en humanos. En la figura 1.8 se muestra un modelo de los diferentes procesos celulares que ocurren durante la infeccion por rotavirus y que finalmente son responsables de la diarrea.

Durante el proceso de infección y replicación de rotavirus en los enterocitos maduros del intestino delgado se produce un aumento de calcio intracelular en el que la proteína NSP4 se encuentra implicada. Este aumento de calcio intracelular en la célula infectada activa un gran numero de procesos intracelulares, incluyendo la desestabilización de la estructura del citoesqueleto celular, la disminución de la expresión de disacaridasas y otras enzimas en la porción apical de las células, la inhibición general de los sistemas de cotransporte de solutos acoplados a Na+ y una reducción de la expresión de las enzimas digestivas en la superficie apical de las células (Ramig, 2004). La proteína NSP4 es secretada de las células infectadas antes de que ocurra la lisis celular (Zhang et al., 2000) e interaccionaría con las células adyacentes no infectadas, produciendo un incremento de calcio intracelular por medio de la activación de la fosfolipasa C (Dong et al., 1997). Este incremento de calcio intracelular provoca la salida de iones cloruro y agua al medio extracelular. Al mismo tiempo, la proteína NSP4 actuaría desestabilizando las uniones célula-célula (“tight junctions”) provocando cambios en la permeabilidad en el epitelio intestinal (Tafazoli et al., 2001). Finalmente la proteína NSP4 o alguna otra molécula efectora secretada por las células infectadas actuarían activando el sistema nervioso entérico, siendo esta activación la responsable de más del 60% del total de la diarrea inducida por rotavirus (Lundgren et al., 2000).

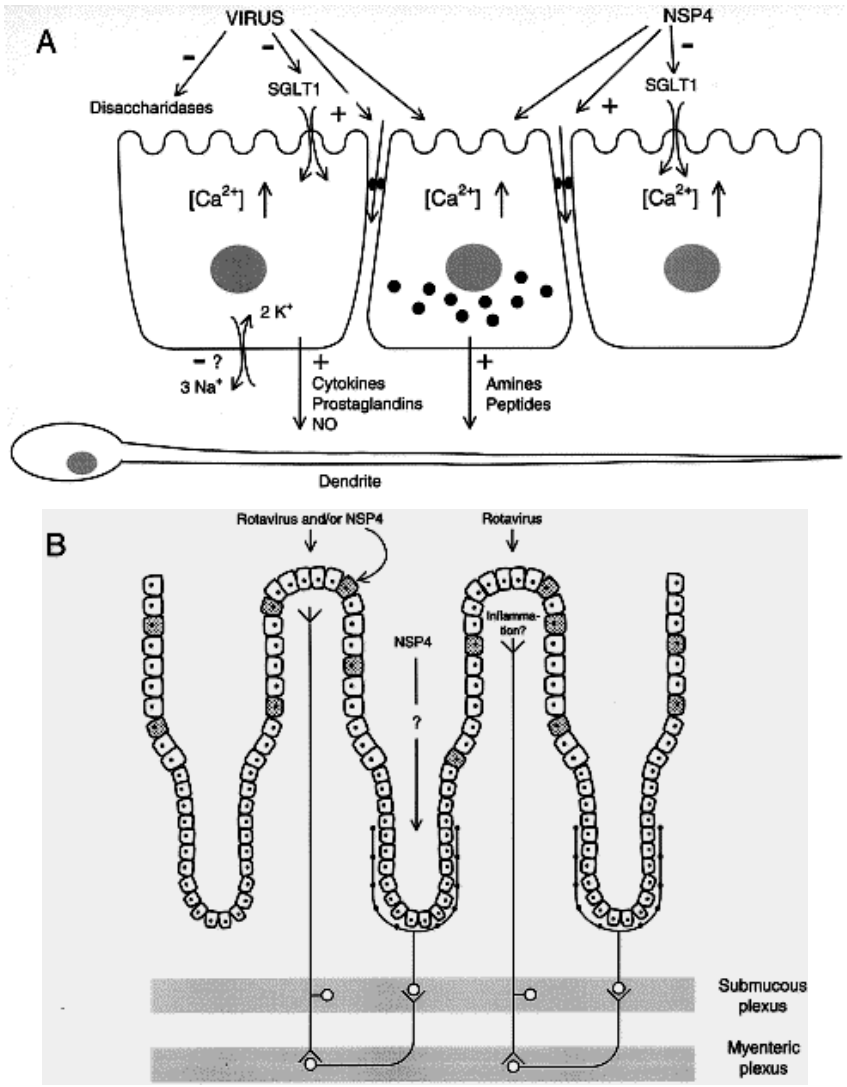

Figura 1.8. Representación esquemática de la inducción de la diarrea por rotavirus. El panel A muestra los cambios a nivel celular que ocurren tanto en los entericitos maduros infectados por rotavirus (izquierda) como en entericitos no infectados (derecha) y en células de la cripta (centro). Rotavirus o su toxina NSP4 inhiben el transporte de electrolitos tras atenuar la activación del simportador sodio-glucosa (SGLT1). Al mismo tiempo la actividad de las disacaridasas es atenuada. Probablemente la actividad de la bomba sodio potasio es también atenuada en la pared basolateral de las células. La permeabilidad intercelular también se ve incrementada por la acción de la proteína NSP4 y/o la infección por rotavirus. Simultáneamente el incremento en la concentración de calcio intracelular tras la infección por rotavirus provoca la secreción de citoquinas, prostaglandinas y óxido nítrico en los entericitos maduros así como de aminas y péptidos en las células de la cripta (aunque algunos de estos efectos están aun por demostrar). Todos estos compuestos biológicamente activos actúan activando las dendritas de las neuronas situadas justo en la siguiente capa de la mucosa y por tanto estimular una respuesta del sistema nervioso entérico. El panel B representa una imagen integrada de los eventos que ocurren a nivel de la mucosa intestinal y que desencadenan la diarrea por rotavirus. Tras el incremento de calcio ocurrido en las células de epitelio la toxina NSP4 y/o la infección por rotavirus activan el sistema nervioso entérico produciendo un reflejo que desencadena la producción de diarrea. (Figura tomada de la referencia Lundgren et al., 2000, con permiso de los autores).

7. Presentación de los trabajos y justificación de la unidad temática

Las publicaciones incluidas en esta Tesis titulada "Estudios sobre la inmunogenicidad y los mecanismos fisiopatológicos de la proteína NSP4 de rotavirus" se dividen en cuatro capítulos:

Capítulo 1. Rodríguez-Díaz, J., López-Andújar, P., García-Díaz, A., Cuenca, J., Montava, R. and Buesa, J. (2003). "Expression and purification of polyhistidine-tagged rotavirus NSP4 proteins in insect cells" Protein Expression and Purification 31: 207-212.

Capítulo 2. Isolation and Caracteritation Single-chain variable fragment (scFv) antibodies against rotavirus NSP4 and VP8* proteins generated by phage display.

- **Capítulo 2A. Rodríguez-Díaz, J**., Monedero,V., Pérez Martínez, G and Buesa, J. (2004). "Single-chain variable fragment (scFv) antibodies against rotavirus NSP4 enterotoxin generated by phage display" Journal of Virological Methods 121(2):231-8.
- **Capítulo 2B.** Monedero, V., **Rodríguez-Díaz, J.,** Viana, R., Buesa, J. and Pérez-Martínez, G. (2004). "Selection of single-chain antibodies against the VP8* subunit of rotavirus VP4 outer capsid protein and their expression in *Lactobacillus casei*". Applied and Environmental Microbiology 70(11):6936-9.

Capítulo 3. Rodríguez-Díaz, J., Montava-Vilaplana, R., García-Díaz, A. and Buesa, J. "Humoral immune response to the NSP4 enterotoxin of rotavirus in Spanish children". Journal of Medical Virology, (2005) submitted (JMVir/2005/6047).

Capítulo 4. Rodríguez-Díaz, J., Banasaz, M; Istrate, C; Buesa, J; Lundgren, O; Espinoza, F; Sundqvist, T; Rottenberg, M and Svensson, L. Nitric oxide production during clinical and experimental infection with rotavirus. Journal of Virology (2005) submitted (JVI00211-05).

Todos los trabajos presentados en la presente Tesis “Estudios sobre la inmunogenicidad y los mecanismos fisiopatológicos de la proteína NSP4 de rotavirus” se centran en describir tanto aspectos inmunogénicos como fisopatológicos de esta proteína no estructural de rotavirus.

En el primer capitulo (“*Expression and purification of polyhistidine-tagged rotavirus NSP4 proteins in insect cells*”) se describe tanto la producción como la purificación de la proteína NSP4 perteneciente a cuatro cepas diferentes de rotavirus en el sistema de expresión de baculovirus. Las proteínas obtenidas de esta forma son la base de los siguientes trabajos presentados en esta Tesis.

En el segundo capítulo (“*Isolation and characterization of single-chain variable fragment (scFv) antibodies against rotavirus NSP4 and VP8* proteins generated by phage display*”) se presentan dos trabajos. En el primero de ellos (Capítulo 2A: “*Single-chain variable fragment (scFv) antibodies against rotavirus NSP4 enterotoxin generated by phage display*”) la técnica de “*phage display*” es utilizada para producir anticuerpos monoclonales de simple cadena frente a la proteína NSP4 de la cepa Wa de rotavirus. La obtención de anticuerpos monoclonales resulta imprescindible a la hora de realizar mapeo epitópico de la proteína, así como para estudiar la posible existencia de epítopos neutralizantes en ella. El primer aspecto se describe en el trabajo 2A, mientras que en el capitulo 2B (“*Selection of single-chain antibodies against the VP8* subunit of rotavirus VP4 outer capsid protein and their expression in Lactobacillus casei*”) se muestra que anticuerpos producidos mediante la misma técnica son capaces de neutralizar la infectividad de rotavirus “in vitro”.

En el capítulo 3 (“Humoral response in spanish population to the NSP4 enterotoxin of rotavirus”) se estudia la relevancia de la proteína NSP4 a la hora de

despertar una respuesta inmune durante la infección de rotavirus “in vivo”, así como la prevalencia y el título de anticuerpos existentes tanto en niños sanos (susceptibles en principio a la diarrea por rotavirus) como en adultos sanos (resistentes a la diarrea por rotavirus).

En el último capítulo de la Tesis (capítulo 4 *“Nitric oxide production during clinical and experimental infection with rotavirus”*) se describe un novedoso aspecto en la fisiopatología de rotavirus como es la participación del óxido nítrico en la fisiopatología de la infección por rotavirus y cómo la proteína NSP4 regula directamente su secreción “in vitro”.

Únicamente en el trabajo 2B (“Selection of single-chain antibodies against the VP8* subunit of rotavirus VP4 outer capsid protein and their expression in Lactobacillus casei”) Jesús Rodríguez Díaz no aparece como primer autor. Su contribución en dicho trabajo consistió tanto en la caracterización de los anticuerpos producidos mediante la técnica de phage display como en la realización de los estudios de neutralización de la infectividad de rotavirus “in vitro”, así como en la posterior caracterización de los anticuerpos producidos en Lactobacillus casei.

OBJETIVOS

Objetivos

La proteína NSP4 de rotavirus ha adquirido un gran protagonismo en los mecanismos fisiopatológicos que se desarrollan en el curso de las infecciones entéricas por rotavirus al haber sido descrita como la primera toxina de origen vírico. Los objetivos de la presente Tesis han sido:

1. Producir y purificar la proteína NSP4 de rotavirus procedente de diferentes cepas, tanto humanas como animales, en el sistema de expresión de baculovirus.

2. Obtener anticuerpos monoclonales necesarios para la caracterización de epítopos de la proteína e identificación de posibles epítopos neutralizantes.

3. Estudiar la respuesta inmune que la proteína NSP4 de rotavirus induce en el transcurso de la infección natural por rotavirus y en la población susceptible y no susceptible a la diarrea por rotavirus.

4. Analizar la capacidad de rotavirus y de la proteína NSP4 de estimular la síntesis de óxido nítrico, tanto “in vitro” como “in vivo”.

CAPÍTULO 1

Expresion and purification of polyhistidine-tagged rotavirus NSP4 proteins in insect cells

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ABSTRACT

The rotavirus nonstructural NSP4 protein, a transmembrane endoplasmic reticulum specific glycoprotein, has been described as the first viral enterotoxin. Both purified NSP4 or a peptide corresponding to NSP4 residues 114-135 induce diarrhea in young mice. NSP4 has a membrane-destabilizing activity and causes an increase in intracellular calcium levels and chloride secretion by a calcium-dependent signalling pathway in eucaryotic cells. In this study four recombinant baculoviruses were generated expressing the rotavirus NSP4 glycoprotein from the human strains Wa and Ito, the porcine strain OSU, and the simian strain SA11, which belong to two different NSP4 genotypes, A and B. The recombinant glycoproteins, expressed as polyhistidine-tagged molecules, were analyzed by Western blotting and immunoprecipitation. Newborn mice responded with diarrhea after inoculation with each of the recombinant NSP4 proteins.

Key words: rotavirus; NSP4; baculovirus; insect cells

Abbreviations used: cAMP, cyclic adenosine monophosphate; ER, endoplasmic reticulum; m.o.i., multiplicity of infection; RT-PCR, reverse transcription and polymerase chain reaction; VLPs, virus-like-particles.

INTRODUCTION

Rotaviruses are the main etiologic agents of viral gastroenteritis in infants and young children worldwide, and cause high childhood mortality in developing countries [1]. Despite extensive studies of different animal models, our understanding of the basic mechanisms of the pathogenesis and immune response in rotavirus infections remains incomplete. Rotavirus infects the mature enterocytes in the mid and upper part of the villi of the small intestine, which ultimately causes diarrhea [2]. The rotavirus nonstructural glycoprotein NSP4 plays a role in viral assembly by acting as an intracellular receptor that mediates the acquisition of a transient membrane envelope as subviral double-layered particles bud into the endoplasmic reticulum (ER) [3]. The 17-20 amino acids from the C-terminus extreme are necessary and sufficient for inner capsid particle binding and this binding is cooperative [4]. NSP4 causes membrane destabilization associated with a region of the protein located within residues 48-91, which includes a potential cationic amphipatic helix [5]. It is postulated that NSP4 acts as a viral enterotoxin and causes diarrhea by triggering a signal transduction pathway in intestinal epithelial cells which increases intracellular calcium, stimulating cAMP-dependent Cl- secretion through a Ca+-dependent signalling pathway [6]. This calcium is mobilized from the ER after activation of phospholipase C [7]. Purified NSP4 or a peptide corresponding to NSP4 residues 114-135 induce diarrhea in young mice, suggesting a role for NSP4 in rotavirus pathogenesis [6, 8]. Furthermore, it has been shown that NSP4 cause diarrhea in newborn mice lacking the cystic fibrosis transmembrane conductance regulator (CFTR), which is thought to modulate epithelial sodium channel function, indicating that NSP4 represents a novel secretory agonist [9]. The humoral and cell-mediated immune responses to NSP4 can be important in the protection against rotavirus infection. Other nonstructural proteins, such as the NSP2 protein, elicit specific antibodies production [10]. It has been shown that NSP4 induces both humoral and cell-mediated immune responses in humans [11]. When focusing on

the role that NSP4 can play in the induction of a protective immune response to rotavirus it is important to compare the deduced amino acid sequences of the different genotypes and to identify conserved motifs. Recent genetic studies revealed the presence of four distinct NSP4 alleles, genetic groups or genotypes (A, B, C and D) including animal and human strains [12, 13, 14, 15]. NSP4 genotype A includes human, bovine and simian strains; NSP4 genotype B includes human and porcine strains, genotype C includes human, simian, canine and feline strains, while genotype D is composed of murine strains [13]. In this study, four recombinant baculoviruses were generated expressing the NSP4 rotavirus protein from the human Wa and Ito strains (both belonging to the NSP4 genotype B), from the simian SA11 strain (NSP4 genotype A), and from the porcine OSU strain (NSP4 genotype B). The recombinant glycoproteins were analyzed by SDS-PAGE and Western blotting and were confirmed to have enterotoxigenic activity in suckling mice.

MATERIALS AND METHODS

Virus and cells

Simian rotavirus strain SA11 (P5B[2], G3) and human strain Wa (P1A[8], G1) were obtained from the American Type Culture Collection (ATCC), porcine rotavirus strain OSU (P9[7], G5) and human rotavirus strain Ito (P1A[8], G3) were kindly provided by Dr. Albert Bosch, University of Barcelona. These four strains were propagated in confluent MA104 cells in the presence of 1 μg/ml trypsin (type IX, Sigma). Spodoptera frugiperda Sf9 insect cells (ATCC-CRL-1711) were grown in Grace's medium (Invitrogen Life Technologies) and used to generate recombinant baculoviruses expressing rotavirus NSP4 proteins by applying the Bac-to-Bac Baculovirus Expression System (Invitrogen Life Technologies).

Cloning of rotavirus NSP4 cDNA into the baculovirus expression vector

Viral RNA was obtained from rotavirus-infected MA104 cells as previously described [16]. The full length NSP4 gene (genomic segment 10) was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using the following primers: sense (5' GGC TTT TAA AAG TTC TGT TCC GAG 3') and antisense (5' GGT CAC ATC AAG ACC ATT CC 3'). The attB1 and attB2 sequences were incorporated into the oligonucleotide primers used for cloning the NSP4 gene in the pDest10 vector using the Gateway system (Invitrogen Life Technologies). Primers attB1-NSP4-B (5' GGGG ACA AGT TTG TAC AAA AAA GCA GGG TTC ATG GAT AAG CTT GCC GAC CTC 3') and attB2-NSP4-B (5´ GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC TCA CAT GGA TGC AGT CAC TTC TGA CGG 3') were used for cloning the NSP4 gene from the rotavirus strains Wa, Ito and OSU. Primers attB1-NSP4-A (5' GGGG ACA AGT TTG TAC AAA AAA GCA GGG TTC ATG GAT AAG CTT ACC GAC CTC 3') and attB2-NSP4-A (5´ GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC TTA CAT TGC TGC TGG TTC TCT TGG TTC 3') were employed for cloning the NSP4 gene from the simian rotavirus SA11 strain. A 6xHis tag was added to the N-terminus of each cloned protein. After confirming the correct insertion of the NSP4 cDNA by sequence analysis of the recombinant pDest10 donor plasmid (Invitrogen), E. coli Max Efficiency DH10Bac cells were transformed and plated onto Luria agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 40 μg/ml IPTG and 100 μg/ml Bluo-gal (Invitrogen) following the instructions of the manufacturer (Bac-to-Bac Baculovirus Expression System Instruction Manual). Recombinant bacmids DNAs were isolated from the transformed bacterial cells and used to transfect Sf9 cells with Cellfectin reagent (Invitrogen Life Technologies). Recombinant baculoviruses were harvested from the cell culture medium at 72 h post-transfection and stored at 4 ºC. The viral stocks were amplified by infecting Sf9 cells, incubating at 27 ºC and harvesting virus at 96 h post-infection. Viral titers were determined by plaque assay following standard procedures [17].

Expression and purification of NSP4 proteins in insect cells

Sf9 cells were infected with the recombinant baculovirus at a multiplicity of infection (m.o.i.) of 10. After 48 h incubation, cells from three T75 cell culture flasks (Costar) were harvested by centrifugation at 500 g for 5 min and resuspended in buffer A (phosphate buffer 50 mM NaH2PO4, pH 8, 300 mM NaCl) containing 10 mM imidazole, 1% Triton X-100 and 0.1% Protease Inhibitor Cocktail (Sigma) for lysis. The lysates were diluted two-fold with buffer A and clarified by centrifugation at 22.000 g at 4ºC for 5 min. The recombinant 6xHis-tagged NSP4 proteins produced in the baculovirus-infected SF9 cells were purified using nickel-nitrile acetate (Ni-NTA) agarose resin (Qiagen). Clarified lysates were incubated with 0.5 ml Ni-NTA resin 30 min at 4ºC with orbital shaking at 200 rpm. The resin was then packed into a Poly-Prep Chromatography Column (Bio-Rad), washed with 3 ml buffer A containing 20 mM imidazole and afterwards with 3 ml buffer A, 50 mM imidazole. Proteins were eluted with 3 ml buffer A, 250 mM imidazole, collecting 0.5 ml fractions. Protein concentrations in these fractions were quantified in a spectrophotometer at 595 nm using the Bradford reagent (Bio-Rad), analyzed by SDS-PAGE and stained with Coomassie blue. The NSP4 protein (28 kDa) was collected in the 250 mM fraction. Fractions containing NSP4 protein were dialyzed against PBS for 24 hours (Membra-Cel dialysis tubing, Serva) and the dialyzed protein was analyzed by SDS-PAGE and Western blotting.

Scale-up of protein production

Two different strategies were used to scale-up NSP4 protein production. In “method A”, increasing amounts of recombinant baculovirus-Sf9 infected cells were lysed as described above. Clarified cell lysates obtained from 3, 6, 12 and 24 T75 cell culture

flasks were mixed and incubated with 0.5 ml, 1 ml, 2 ml and 4 ml of Ni-NTA resin, respectively, for 30 min with shaking at 200 rpm at 4ºC. The resin was centrifuged at 120 g for 5 min and the beads were transferred to four chromatography columns. Beads were washed and protein was eluted as described above. In “method B”, clarified cell lysates were obtained from sets of three T75 cell culture flasks containing recombinant baculovirus-Sf9 infected cells and each lysate was incubated with 0.5 ml Ni-NTA. Beads from lysates were pooled corresponding to the equivalent of 3, 6, 12 and 24 T75 cell culture flasks and the mixtures, consisting of volumes of 0.5, 1 ml, 2 ml, and 4 ml resin suspensions, were packed separately into chromatography columns for the washing and elution steps.

Western blotting of NSP4 glycoproteins

Eluted fractions were analyzed by SDS-PAGE using 10% gels. The proteins were transferred onto nitrocellulose using Tris-glycine transfer buffer at 30 V overnight. NSP4 glycoproteins were detected by an anti-NSP4 (114-134) rabbit polyclonal antiserum (1/1,000), and with an anti-NSP4 mouse polyclonal antiserum raised in our laboratory (1/1,000). Peroxidase-labeled anti-rabbit and anti-mouse antibodies were used as secondary antibodies (1/6,000) and 3-amino-9-ethylcarbazole was employed as the substrate (Sigma Immunochemicals).

Glycosylation status of NSP4 and deglycosylation analysis

The glycosylation status of the recombinant NSP4Wa protein was confirmed by PAGE analysis, followed by Western blotting with ConA peroxidase. The nitrocelullose membrane was blocked with 3% bovine seroalbumin (BSA) in PBST (PBS, 0.2% Tween 20), and incubated with 5 μg/ml ConA peroxidase in PBST at room temperature for 1 h. Purified NSP4Wa protein was incubated at 37 ºC for 72 h with 0.01 units of

endo-β-N-acetylglucosaminidase-H (Endo-H, Roche) in 150 mM sodium citrate buffer, pH 5.5, to analyse its sensitivity to this enzyme.

Diarrhea induction by NSP4

The ability of the different recombinant NSP4 proteins to induce diarrhea in young mice was assayed by inoculating intraperitoneally 1 nmol of each protein in 50 μl of sterile PBS per dose as previously described [6]. Groups of 10 5-day-old Balb/c mice were inoculated with NSP4 encoded by gene 10 from rotavirus strains Wa, Ito, OSU and SA11. One group of mice was inoculated with 1 nmol of a Sf9 cell 90 kDa protein in 50 μl of sterile PBS as a negative control. Mice were observed hourly for 8 hours.

RESULTS

Expression and purification of NSP4 proteins in insect cells

We constructed recombinant baculoviruses expressing the NSP4 protein from four different rotavirus strains representing two distinct NSP4 genotypes, A and B. The expression of $NSP4_{Wa}$, $NSP4_{Ito}$, $NSP4_{OSU}$ and $NSP4_{SA11}$ by the recombinant baculoviruses showed low differences in protein production levels, although immunoblots of cellular lysates revealed a major production of $NSP4_{SA11}$ (Fig. 1). SDS-PAGE analysis and Western blotting showed two major bands corresponding to monomers of NSP4, with molecular wights of 21kD and 28 kD. Oligomers of the protein were also detected. NSP4 without post-translational modifications has an apparent molecular weight of 21kD, and of 28 kD when glycosylated. Different patterns of glycosylation were observed, and there is an incomplete post-translational glycosylation in insect cells. The $NSP4_{Wa}$ protein was the most efficiently glycosylated,

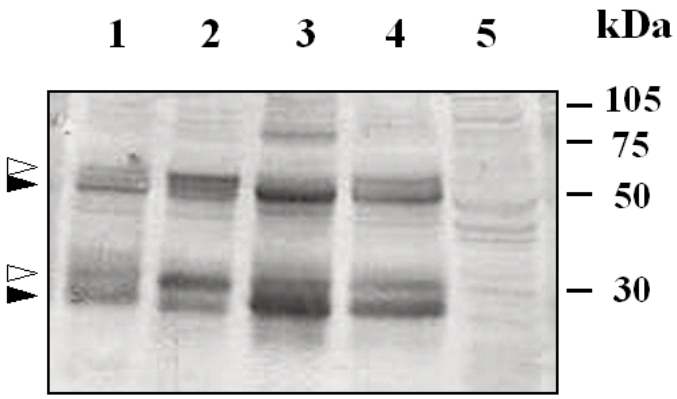

Fig. 1. Western blot analysis of NSP4 proteins produced in Sf9 cells infected with recombinant baculoviruses. Lane 1, OSU NSP4; lane 2, Wa NSP4; lane 3, SA11 NSP4; lane 4, Ito NSP4, and lane 5, uninfected Sf9 cells. Monomers and dimers of the NSP4 proteins can be seen in each lane. Open arrowheads indicate glycosylated NSP4; closed arrowheads indicate unglycosylated NSP4.The molecular size markers in kDa are indicated.

whereas $NSP4_{OSU}$ and $NSP4_{Ito}$ showed less than 50% glycosylation. $NSP4_{SA11}$ was poorly glycosylated (less than 20%) (Fig. 1). Production of NSP4 was in the range of 50-100 μg/ml in the second 250 mM imidazole fractions. These fractions were quantified by the Bradford method and analyzed by SDS-PAGE and Western blotting (Fig. 2). Complete purity of the NSP4 proteins could not be achieved in our study because a 90 kDa cellular protein co-purified in the chromatography eluate.

Glycosylation status of NSP4 and deglycosylation analysis

To confirm that the recombinant NSP4 proteins are glycosylated, they were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with ConA peroxidase. The purified NSP4 proteins expressed by recombinant baculoviruses were found to be glycosylated (Fig. 3, panel A). Deglycosylation analysis with endo-H confirmed the glycosylated status of the $NSP4_{SA11}$ protein (Fig. 3, panel B).

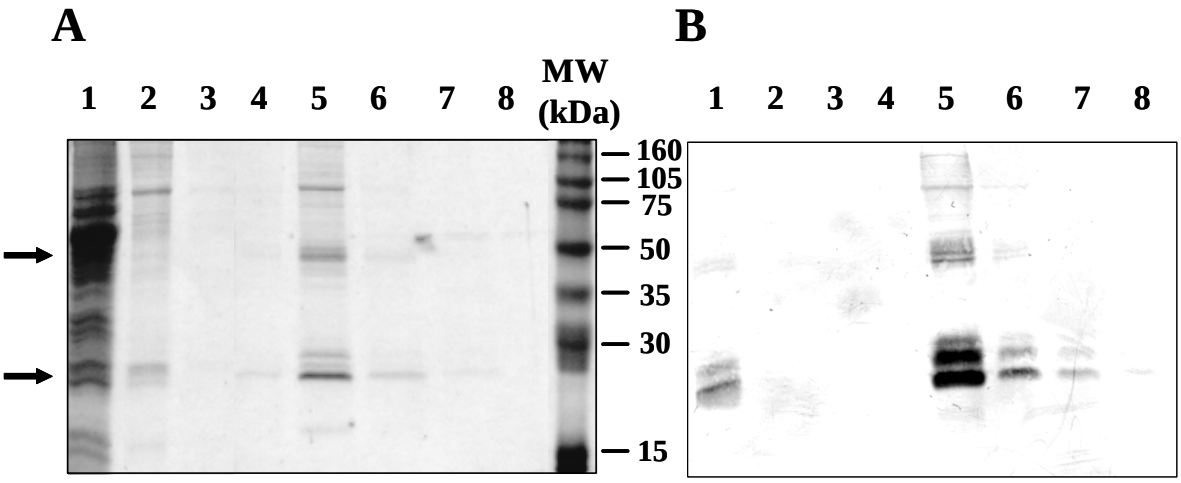

Fig. 2. Purification of $NSP4_{SA11}$ protein from recombinant baculovirus-infected SF9 cells. Lane 1, clarified lysate of baculovirus-infected Sf9 cells. The purification was carried out as described in Materials and methods. Eluted fractions were collected and analysed by SDS-PAGE (10%). Panel A shows a Coomassie blue stained gel and panel B represents a Western blot of the same. Lanes 2 and 3, Ni-NTA agarose flow-through fractions corresponding to the 50 mM imidazole elution buffer. Lanes 4 to 8, flow-through fractions corresponding to the 250 mM imidazole elution buffer. The NSP4 protein (28 kDa) was collected in the first 250 mM fraction. Arrows show purified NSP4. The molecular size markers in kDa are indicated.

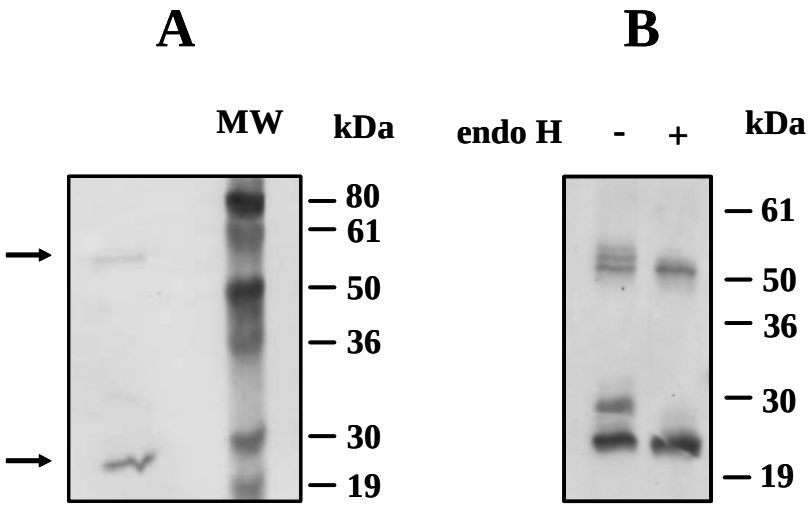

Fig. 3. Glycosylation status of $NSP4_{SA11}$. The purified $NSP4_{SA11}$ protein was resolved by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with ConA-peroxidase (panel A). The purified $NSP4_{SA11}$ protein expressed by the recombinant baculovirus was stained with ConA-peroxidase, both as monomeric (lower arrow) and oligomeric forms (upper arrow). Deglycosylation analysis with endo-H confirmed the glycosylated status of the $NSP4_{SA11}$ (panel B).

Scale-up NSP4 protein production

Scaled-up production of NSP4 following two different protein purification strategies showed a proportional increase of NSP4 recovery with both methods assayed. A higher protein yield was achieved by incubating the clarified Sf9 cell lysates from 3 T75 cell culture flasks with 0.5 ml of Ni-NTA resin and packing the beads accrued from 3, 6, 12 and 24 flasks ("method B") than mixing first the clarified Sf9 cell lysates obtained from the same number of flasks and incubating the mixtures afterwards with proportionally increased volumes of Ni-NTA resin ("method A") (Fig. 4).

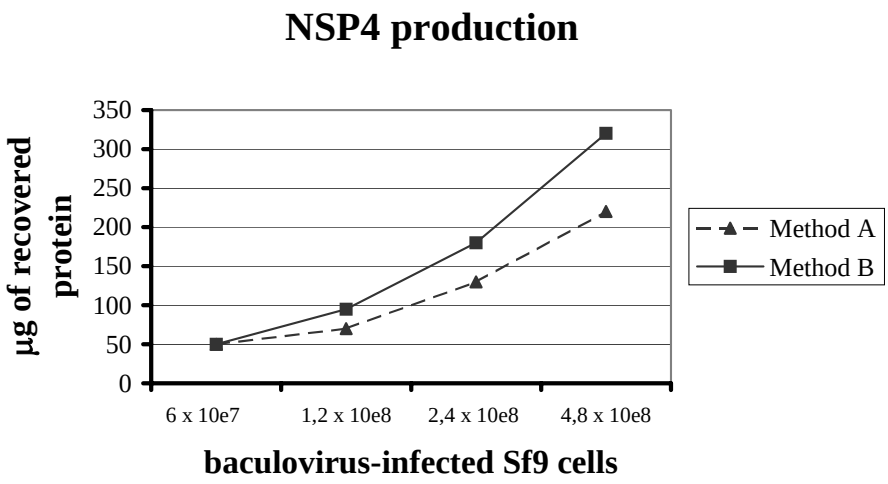

Fig. 4. Comparison of NSP4 production by two different procedures: in method A (mixed clarified Sf9 cell lysates) cell lysates from 3, 6, 12 and 24 T75 cell culture flasks containing recombinant baculovirus-Sf9 infected cells were mixed and incubated with 0.5, 1, 2 and 4 ml of Ni-NTA resin, respectively. The resin was centrifuged and transferred to four chromatography columns (Bio-Rad), washed and the protein eluted. In method B (mixed beads), clarified cell lysates from sets of three T75 cell culture flasks containing recombinant baculovirus-Sf9 infected cells were incubated with 0.5 ml Ni-NTA resin. The beads from lysates derived from 3, 6, 12 and 24 T75 cell culture flasks were collected together, and each mixture was packed separately into a chromatography column for the washing and elution steps as described in Materials and methods.

Diarrhea induction by NSP4

The induction of diarrhea by the recombinant NSP4 proteins was studied in newborn Balb/c mice. All the mice developed diarrhea in the next four hours when they were given NSP4 corresponding to rotavirus strains Wa, Ito, OSU and SA11. No mice responded with diarrhea when they were inoculated with the negative control (Table 1).

Only intraperitoneal inoculation of NSP4, but not oral inoculation, induced diarrhea in suckling mice, as previously reported [6].

Table 1. Diarrhea induced by intraperitoneal administration of purified NSP4s from different rotavirus strains in newborn mice.

Protein given ([a])	**Diarrhea outcome** ([b])
$NSP4_{Wa}$	10/10
$NSP4_{Ito}$	10/10
$NSP4_{OSU}$	10/10
$NSP4_{SA11}$	10/10
Sf9 cell protein ([c])	0/10

[a]1 nmol of each recombinant NSP4 protein diluted in 50 μl PBS was given intraperitoneally to newborn Balb/c mice.
[b]No. of mice suffering diarrhea/No. of challenged mice.
[c]1 nmol of a Sf9 cell 90 kDa protein diluted in 50 μl PBS was given intraperitoneally to newborn Balb/c mice as a negative control.

DISCUSSION

The present paper describes the expression, purification and biological activity of recombinant rotavirus nonstructural NSP4 proteins from four different viral strains using the baculovirus expression system. The rotavirus NSP4 protein has been proven to be a viral protein of great interest according to its biological and enterotoxigenic properties. This protein consists of 175 amino acids and contains sequences responsible for the membrane-destabilizing and enterotoxic effects that have been mapped to different regions of the polypeptide [5]. It has been shown that the enterotoxin domain is highly conserved among avian and mammalian rotaviruses in terms of its sequential and structural properties, independently of the NSP4 genotype [18]. Our goal was to express and purify recombinant NSP4 proteins from the human Wa and Ito strains (both belonging to the NSP4 genotype B), from the simian SA11 strain (NSP4 genotype A), and from the porcine OSU strain (NSP4 genotype B) to

investigate whether the amino acid differencies in their sequences may be responsible for differences in their enterotoxigenic activities. In this study we show that all four recombinant NSP4 proteins have enterotoxigenic activity in suckling mice when given intraperitoneally, what suggests that this activity must be important as a pathogenetic mechanism in rotavirus infections.

The baculovirus-insect cell expression system, rather than bacterial expression, was chosen to express the NSP4 proteins because they are glycosylated proteins, with two potential N-glycosylation sites in the NSP4 molecule of all mammalian strains. However, it has been recently reported that purified NSP4s of avian rotaviruses (PO13, Ty-1, Ty-3 and Ch-1) expressed in E. coli had enterotoxigenic activities in suckling mice [18].

The expression of NSP4 in Sf9 cells has been previously shown to cause an increase in intracellular calcium levels [Ca2+]i [7]. Paradoxically, it has been demonstrated that the expression of NSP4 in Spodoptera cells do not influence the permeability of the plasma membrane, but alters plasma membrane permeability in mammalian cells [5, 19, 20]. Although very high concentrations of NSP4 are cytotoxic to Sf9 cells, levels normally found in rotavirus-infected cells do not affect cell viability [20].

Polyhistidine-tagged NSP4 production in insect cells allows the purification of this protein following a fast and easy procedure. NSP4 is an integral membrane protein resident in the ER with two N-linked oligosaccharide sites at the amino-terminus, which is on the luminal side of the ER. It has been reported that the membrane association properties of NSP4 make purification of this protein difficult, although it can be achieved by immunoaffinity chromatography [20]. By using the strategy described in this study, 6xHis-tagged NSP4 can be produced and purified easily with a one-step Ni-NTA agarose chromathography. Furthermore, histidine tags can be cleaved with rTEV protease cleavage and removed by affinity chromatography.

Antibodies to NSP4 may play a role in the protection against rotavirus infections. It has been shown that NSP4 induces both humoral and cellular immune responses in

humans [11], and elicits systemic and intestinal antibody responses in a gnotobiotic pig model of human rotavirus disease [21].

The recombinant proteins produced in this study are currently being used as antigens to detect specific antibodies against NSP4 in human sera from children convalescing from acute rotavirus infection and in different groups of individuals. They are also invaluable tools to study their effect on the mouse intestinal mucosa and to analyze the role that anti-NSP4 antibodies can play in the protection against rotavirus infection in the mouse model. Many aspects of the function of NSP4 in viral morphogenesis, pathogenesis and immunity still remain to be clarified.

It has been highlighted the therapeutic potential that synthetic analogs of the active NSP4114-135 peptide may have for the treatment of neonatal meconium ileus syndrome or to counteract chronic constipation in cystic fibrosis patients [9]. With these purposes, further studies on the molecular characterization of this protein are needed using purified recombinant NSP4 proteins or peptides.

ACKNOWLEDGMENTS

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CAPÍTULO 2

Isolation and characterization of single-chain variable fragment (scFv) antibodies against rotavirus NSP4 and VP8 proteins generated by phage display*

CAPÍTULO 2A

Single-chain variable fragment (scFv) antibodies against rotavirus NSP4 enterotoxin generated by phage display

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Single-chain variable fragment (scFv) antibodies against rotavirus NSP4 enterotoxin generated by phage display

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Abstract

The rotavirus non-structural NSP4 protein causes membrane destabilization as well as an increase in intracellular calcium levels in eukaryotic cells and induces diarrhea in young mice, acting as a viral enterotoxin. In this study the phage display technique was used to generate a panel of single-chain variable fragment (scFv) antibodies specific for the NSP4 protein of the human rotavirus strain Wa from a human semi-synthetic scFv library. After several rounds of panning and selection on NSP4 adsorbed to polystyrene tubes, individual scFv were isolated and characterized by fingerprinting and by sequencing the VH and VL genes. The isolated scFv antibodies specifically recognize NSP4 in enzyme immunoassay and in Western blot. Four truncated forms of the NSP4 protein were constructed which allowed us to map the binding region of the selected scFv antibodies to the C-terminal portion of NSP4. The isolated scFv antibodies constitute valuable tools to analyse the mechanisms of NSP4 functions.

Keywords: Rotavirus; NSP4; Phage display library; Single-chain variable fragment (scFv) antibodies. Abbreviated article title: scFv antibodies against NSP4

1. Introduction

Rotaviruses (RV) are the main etiologic agents of viral gastroenteritis in infants and young children worldwide, producing high childhood mortality in developing countries (Parashar et al., 2003). Rotaviruses infect mature enterocytes in the mid- and upper parts of the villi of the small intestine, which ultimately causes diarrhea (Lundgren and Svensson, 2001). The rotavirus non-structural glycoprotein NSP4 is an intracellular receptor that mediates the acquisition of a transient membrane envelope as sub-viral particles bud into the endoplasmic reticulum. NSP4 acts as an intracellular receptor for double-layered particles (DLPs) of rotavirus, interacting with VP6 (Estes, 2001; 2003). Many structural motifs or protein regions have been implicated in the NSP4 biological function. Amino acids 17 to 20 from the C-terminus extreme are necessary and sufficient for inner capsid particle binding (O'Brien et al., 2000) and the region involved in the retention of the NSP4 protein into the endoplasmic reticulum has been mapped between the amino acids 85 and 123 in the cytoplasmatic region of the protein (Mirazimi et al., 2003). Residues at positions 48 to 91, a region which includes a potential cationic amphipatic helix, have been shown to be involved in membrane destabilization (Tian et al., 1996; Browne et al., 2000). Purified NSP4 or a peptide corresponding to NSP4 residues 114-135 induce diarrhea in young mice after an increase in intracellular calcium levels, suggesting a role for NSP4 in rotavirus pathogenesis (Tian et al., 1994; Ball et al., 1996; Horie et al., 1999).

Six distinct alleles, genotypes or genetic groups (A through F) including animal and human strains have been described for the NSP4 protein (Ciarlet et al., 2000; Cunliffe et al., 1997; Kirkwood et al., 1997; Cao et al., 1999; Borgan et al., 2003). Genotype A includes human, bovine and simian strains; genotype B includes human and porcine strains; genotype C includes human, simian, canine and feline strains; genotype D includes murine strains (Ciarlet et al., 2000), while genotypes E and F are composed of avian strains (Mori et al., 2002).

The significance of the humoral and cell-mediated immune responses to NSP4 in the protection against rotavirus infection in humans is still unknown. Other non-structural proteins, such as the rotaviral NSP2 protein, elicit the production of specific IgG antibodies (Colomina et al., 1998). It has been shown that NSP4 induces both humoral and cell-mediated immune responses in humans (Johansen et al., 1999; Ray et al., 2003). However, very few studies have been carried out to elucidate the immunologic determinants in the NSP4 protein. Recently two immunodominant regions have been described in the NSP4 of group A avian rotavirus PO-13 (P[17], G7, NSP4 genotype E). The antigenic site I (AS I) (amino acids 151 to 169) and the antigenic site II (AS II) (amino acids 136 to 150) are the two immunodominant regions and are located in the cytoplasmatic tail (Borgan et al., 2003).

To increase our knowledge on the NSP4 protein and to elucidate the effects that blocking of some specific epitopes of this protein can have in rotavirus pathogenesis, it is necessary to obtain and characterise monoclonal antibodies against NSP4. Single-chain variable fragment (scFv) antibodies can be particularly useful for performing this type of studies. scFv are chimeric proteins produced by a fusion of the heavy V_H and the light V_L variable regions of immunoglobulins and have epitope recognition sites similar to monoclonal antibodies. scFv libraries of randomly combined V_H and V_L chains from immune or non-immune individuals are usually fused to the minor coat protein of M13 filamentous phage (pIII). This allows display of the scFv proteins on the M13 surface and selection of scFv directed to a particular antigen by several rounds of panning on immobilized antigen (Harrison et al., 1996; Griffiths and Duncan, 1998). This technology is used for the isolation of monoclonal antibody reagents bypassing hybridoma technology. In this study the phage display technique was used to generate a panel of scFv antibodies against NSP4 from the Griffin.1 library. Four truncated forms of NSP4 were constructed in order to elucidate the region recognized by individual

antibodies. The isolated scFv antibodies can be used to further analyse the NSP4 biological functions, particularly its effect as an enterotoxin.

2. Materials and methods

2.1. Expression and purification of NSP4 proteins in insect cells

Two recombinant baculoviruses expressing the NSP4 glycoproteins from the simian rotavirus strain SA11 (P5B[2], G3, NSP4 genotype A) and the human strain Wa (P1A[8], G1, NSP4 genotype B) were used to produce the recombinant NSP4 glycoproteins (Rodriguez-Diaz et al., 2003). Spodoptera frugiperda 9 (Sf9) cells were infected with the recombinant baculoviruses at a multiplicity of infection (m.o.i.) of 10 and incubated for 48 h. The recombinant proteins were purified by affinity chromatography as previously described (Rodriguez-Diaz et al., 2003). Complete purity of the NSP4 proteins could not be achieved because a 90 KDa cellular protein co-purified in the chromatography eluate. Proteins were dialysed and analysed by SDS-PAGE and their concentrations quantified by the Bradford method.

2.2. Cloning, expression and purification of GST- NSP4 truncated proteins in E. coli

Four truncated forms of NSP4 from the rotavirus strain Wa were constructed fused to the glutathione S-transferase (GST) according to the structural motifs of the NSP4 protein: GST-NSP4$_{1-50}$, corresponding to amino acids 1 to 50; GST-NSP4$_{1-91}$, corresponding to amino acids 1 to 91; GST-NSP4$_{1-135}$, corresponding to amino acids 1 to 135 and GST-NSP4$_{1-175}$, representing the whole NSP4 protein. The NSP4Wa gene was amplified by PCR with specific primers incorporating NotI and SalI restriction sites

for directional cloning into the pGEX-4T-3 expression vector. Primers were: NSP4-1 (5'-TTT GTC GAC **ATG** GAT AAG CTT GCC GAC) as the forward primer for all reactions and NSP4-50 (5'-TTT GC GGC CGC **TCA** TGA AGC TTT ATG TAA TGT), NSP4-91 (5'-TTT GC GGC CGC **TCA** TGT AGT AAC CTG CTC TTT), NSP4-135 (5'-TTT GC GGC CGC **TCA** TAT CAG GTT GTC ATG TAT), NSP4-175 (5'-TTT GC GGC CGC **TCA** CAT GGA TGC AGT CAC), as the reverse primers, respectively. The restriction sites are underlined and the start and stop codons are indicated in bold. Cultures of E. coli DH5α were transformed with the pGEX-4T-3 derivatives and were then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. Bacterial cells were harvested and pellets were resuspended in a 1/50 volume of PBS. After sonication, Triton X-100 was added to the cell lysate at a final concentration of 1%, and cellular debris was removed by centrifugation at 20.000xg at 4°C. One milliliter of 50% glutathione-Sepharose 4B (Amersham Biosciences) was added to 10 ml of extract supernatant, and the resultant mixture was incubated with gentle agitation for 1 h at room temperature. The mixture was extensively washed with PBS, and proteins were eluted with an equal volume of 10 mM reduced glutathione in 50 mM Tris-HCl pH 8.0.

2.3. Antibody phage display to the NSP4 protein

The Griffin.1 library, which is a scFv phagemid library made from synthetic V-gene segments, was generously provided by Dr. A. D. Griffiths (Centre for Protein Engineering, Medical Research Council, Cambridge, U.K.). The library was made by recloning the heavy and light chains variable regions from the lox library vectors (Griffiths et al., 1994) into the phagemid vector pHEN2. Three rounds of selection were performed following a panning method previously described (Marks et al., 1991). Immunotubes (Nunc Maxisorp) were coated overnight with 4 ml of purified NSP4Wa (10 μg/ml) in 50 mM sodium hydrogen carbonate pH 9.6 at room temperature. The tubes were washed three times with PBS and blocked for 1 h with 2% skim milk in PBS

(M-PBS). Then 10^{12} phage particles in 4 ml of 2% M-PBS were added and allowed to bind for 30 min at room temperature by rotating continuously and then without rotation for at least 90 min. For the first round of selection, tubes were washed 10 times with PBS containing 0.1% Tween-20 (T-PBS) and 10 times with PBS. The following rounds were washed 20 times with T-PBS and 20 times with PBS. Bound phages were eluted by adding 1 ml of 100 mM triethylamine pH 11.6 and rotating 10 min at room temperature. Eluted phages were neutralized with 0.5 ml of 1 M Tris-HCl pH 9.6 and used to infect 10 ml of an exponentially growing E. coli TG1 culture for 30 min at 30 ºC without shaking. The infected culture was pelleted, resuspended in 1 ml of 2xTY, plated on a large Petri dish (Nalge Nunc) of TYE containing 100 μg/ml ampicilin, 1% glucose, and grown overnight at 30 ºC. E. coli TG1 cells were recovered from the plate and infected with VCS-M13 helper phage (Stratagene) at a m.o.i. of 20. Phages were grown and recovered from supernatant by adding 1/5 volume of 20% PEG 6000, 2.5 M NaCl followed by 1 h of incubation at 0 ºC and spinning at 10.800 x g for 10 min. Pellets containing phages were resuspended in 2 ml of PBS.

2.4. Screening of individual phage clones by ELISA

Individual phages obtained in the third round of selection were screened by ELISA. Ninety-six-well ELISA plates (Costar) were coated with 0.1 μg of purified NSP4Wa per well. Proteins extracted from Sf9 insect cells were used as a negative control. ELISA was performed with the total phages obtained in the three rounds of selection and with individual clones selected from the third round of selection. Coated plates were washed and blocked with 2% M-PBS 1 h at room temperature. Phages were added in 100 μl of 2% M-PBS and incubated for 2 h. Plates were washed and a horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal antibody (Amersham Biosciences) 1:5000 diluted in 2% M-PBS was added. After incubation at room temperature for 2 h, plates

were washed and developed by adding 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) as the substrate. Optical density was measured at 450 nm.

2.5. Detection of binding specificity by ELISA

Immunoplates (Nunc) were coated with 0.1 μg of purified NSP4Wa or NSP4SA11 per well to analyse the cross-reactivity of the isolated scFv antibodies. Wells were also coated with 0.1 μg of each of the truncated forms of the NSP4Wa per well. A 90 KDa protein purified from Sf9 cells or purified GST were used as the negative controls. The following steps of the ELISA procedure were performed as described above.

2.6. scFv characterisation

The ELISA-positive clones were characterized by BstNI restriction analysis and fingerprinting as previously described (Clackson et al., 1991). scFv antibody genes were amplified from single E. coli colonies with primers LMB3 (5`-GAGGAA ACAGCTATGAC-3´) and Fd seq 1 (5´-GAATTTTCTG TAT GAGG-3´) followed by digestion of the PCR products with BstNI (Marks et al., 1991). Clones were classified according to the fingerprinting patterns and sequenced with primer FOR_LinkSeq (5´-GCCACCTCCGCCTGAACC-3´) to sequence the VH genes and pHEN-SEQ (5´-CTATGCGGCCCCATTCA-3´) to obtain the sequence of the VL genes. The nucleotide sequences of the PCR products were analyzed using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems).

The reactivity of the scFv antibodies against denatured NSP4Wa protein was evaluated by Western blotting. 100 ng of purified NSP4 were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. Phages displaying scFv were used as first antibodies and HRP-conjugated anti-M13 (Amersham Biosciences) was used as

second antibody. After washings, reactivity was detected with the substrate 3-amino-9-ethylcarbazole (AEC, Sigma).

3. Results

3.1. Isolation of scFv against NSP4

The Griffin.1 phage display library was used to select phage antibodies against purified NSP4 from the human rotavirus strain Wa. Phage suspensions ($5x10^{12}$ to $1x10^{13}$ PFU) were applied, and after each round of panning and selection on NSP4-coated immunotubes the number of eluted phages was determined by plating. Phage titres increased after the third round of selection.

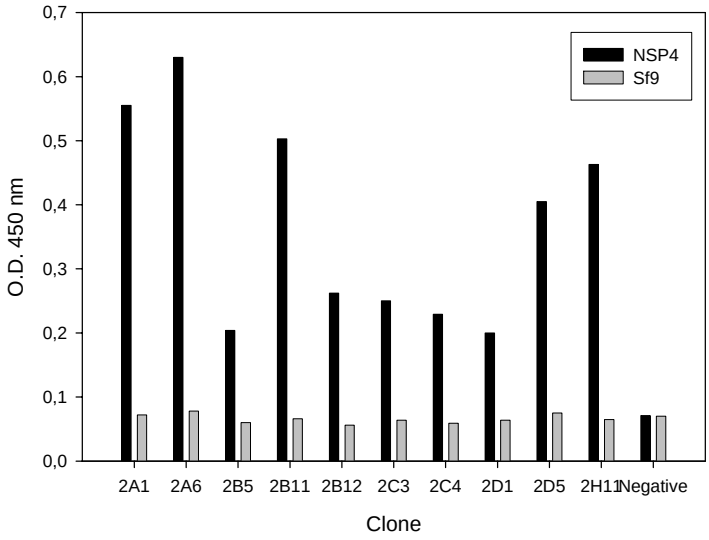

Figure 1. ELISA analysis of ten selected clones of M13 phages expressing scFv from the third round. Selected phages specifically reacted against NSP4 but not against a 90 KDa protein from Sf9 cells that co-purified with the baculovirus-expressed NSP4. A negative clone randomly chosen was used as a control. Phage-infected E. coli TG1 culture supernatants containing ~10^7 phage particles per ml were analysed in the assay.

The enrichment of the phage population in phages specifically recognizing the NSP4 protein was demonstrated by an increase of the reactivity against NSP4 in ELISA with the pool of phages isolated after each round of selection (results not shown). As the NSP4 preparations carried a contaminant protein of 90 KDa derived from Sf9 insect

cells, 96 independent clones from the third round were simultaneously tested by ELISA against NSP4 and against the Sf9 purified protein. Ten clones were selected that specifically reacted against NSP4 but not against the Sf9 protein. These clones were repurified and their specificity was confirmed in a second ELISA test. As shown in Fig. 1, the ten selected phage clones specifically recognized NSP4.

3.2. scFv characterisation

The scFv gene of the selected clones was amplified by PCR and subjected to BstNI restriction analysis. Four different banding patterns were detected (Fig. 2). The presence of four different scFv genes was also confirmed by sequencing the VH and VL regions, which were analysed with the V-BASE database of human immunoglobulin genes (Tomlinson et al., 1992).

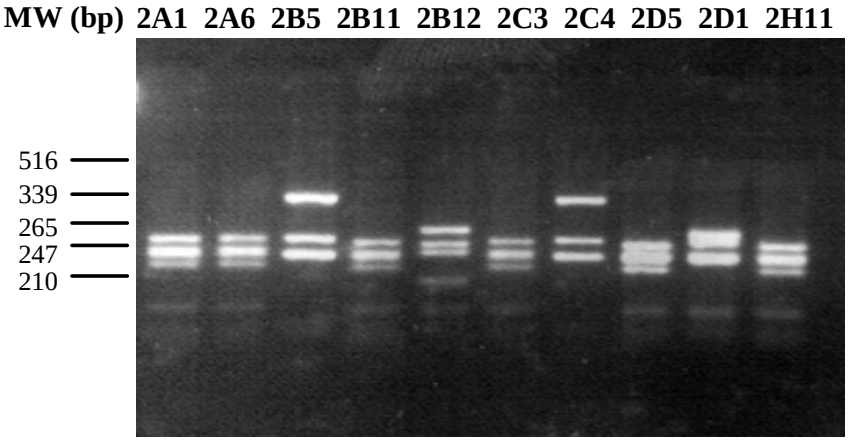

Figure 2. BstNI fingerprinting analysis of the scFv genes present in the selected clones reacting against NSP4. The scFv gene inserts were was amplified by PCR, digested with BstNI and resolved in a 3% agarose gel. Four different banding patterns were detected.

The four established groups contained four different complementarity determining regions (CDR3) in their VH segments (Table 1). Group I was represented by six clones; group II by two clones, while groups III and IV had only one representative. One clone from each groups I and II was chosen (clones 2C3 and 2B5, respectively). The four groups were also evaluated by Western blotting with NSP4 as antigen using HRP-

conjugated anti-M13 as the second antibody (Fig. 3). Clones 2B5, 2C3, and 2D1 were positive by Western blot, while 2B12 gave no signal. This result indicated that three clones recognized linear epitopes that are conserved after protein denaturation, whereas clone 2B12 recognized most likely a conformational epitope in the NSP4 protein.

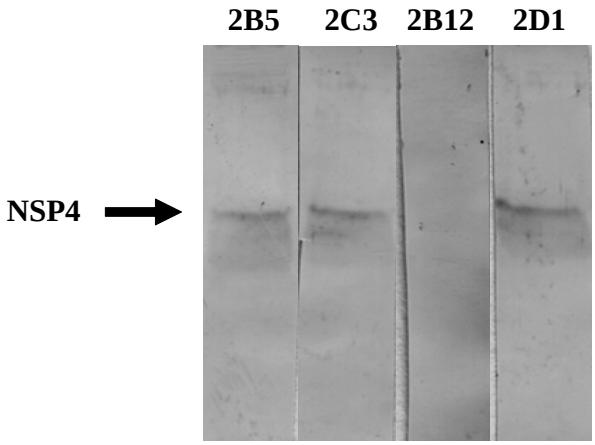

Figure 3. Western blot analysis after SDS-PAGE of $NSP4_{Wa}$ probed with M13 phages bearing anti-NSP4 scFv antibodies fused to the pIII phage capsid protein (i.e., scFv::pIII fusions). The binding was detected with an HRP- conjugated anti-M13 monoclonal antibody.

Table 1. Amino acid sequences of the V_H regions CDR3 of 10 isolated scFv genes containing clones specifically reactive with NSP4.

Clone	CDR3
2A1, 2A6, 2B11, 2C3, 2D5, 2H11	GSGMER
2B5, 2C4	GSIRDPIK
2B12	RLDI
2D1	YTDSRHGGVG

3.3. Detection of binding specificity by ELISA

The ability of each clone to recognize the NSP4 from two different rotavirus genotypes was analysed by ELISA. As shown in Fig. 4, three of the clones (2B5, 2B12 and 2C3) were able to recognize both NSP4 proteins of genotype A (rotavirus strain SA11) and genotype B (rotavirus strain Wa), indicating that they were interacting with conserved

motifs present in the two assayed genotypes. Clone 2D1 only reacted with the NSP4 from genotype B (Fig. 4).

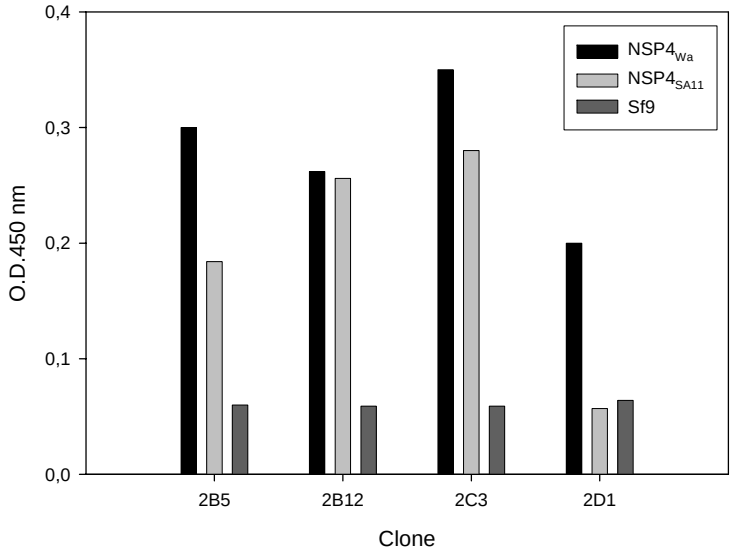

Figure 4. Analysis of scFv antibodies binding specificity by ELISA. Recognition of two different NSP4 genotypes, genotype A (strain SA11) and genotype B (strain Wa), by clones 2B5, 2B12, 2C3 and 2D1. Clone 2D1 only reacted with the NSP4 from genotype B. A Sf9 cellular protein of 90 KDa of MW was used as the antigen in the specificity control.

In order to map the NSP4wa region recognized by each scFv clone, four NSP4 truncated forms were constructed, expressed and purified as GST fusions (Fig. 5) and used as antigens in ELISA tests (Fig. 6). A mouse polyclonal antiserum raised against the NSP4Wa protein strongly reacted only with the C-terminal region of the protein (amino acids 135-175). The region containing the amino acids 91 to 135 was poorly recognized and no signal was detected against the more hydrophobic region of the protein. The scFv clones were specific to only two of the four regions in NSP4: clones 2B5, 2C3 and 2D1 recognized the truncated proteins GST-$NSP4_{1-135}$ and GST-$NSP4_{1-175}$, but not the NSP4 region spanning from amino acids 1 to 91 (Fig. 6). Therefore, these three clones recognized a region of the protein between amino acids 91 and 135, which contains the toxigenic peptide (amino acids 114-135). Clone 2B12 was able to interact only with the full-length NSP4 in the ELISA test (Fig. 6) but did not

recognized this protein by Western blot analysis (Fig. 3). This clone might recognize a conformational epitope in which residues within the last forty amino acids of the NSP4 might be implicated. Table 2 summarizes the reactivity patterns of the isolated scFv, the region of the NSP4 recognized by the scFv clones, their binding to NSP4 by Western blotting and the reactivity against NSP4 genotypes A and B by ELISA.

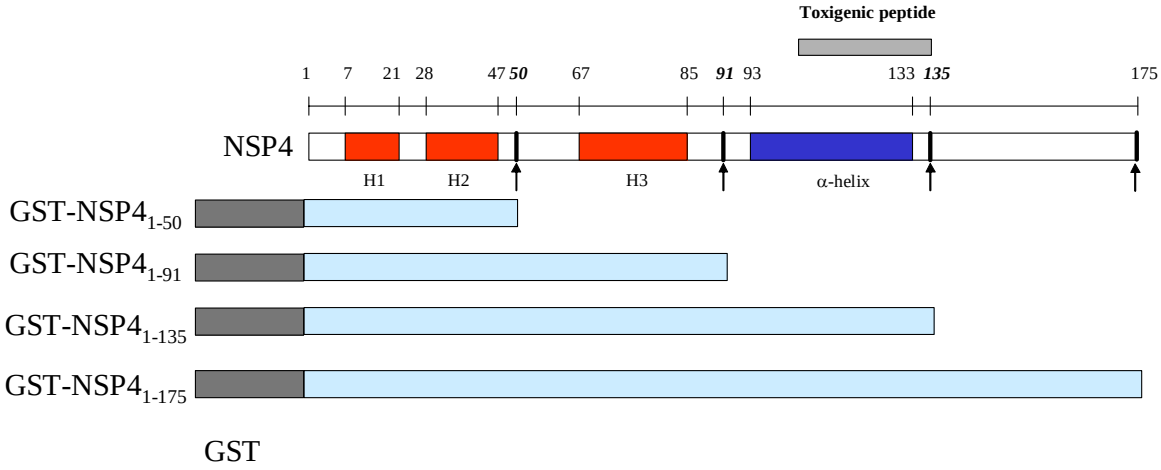

Figure 5. Schematic representation of the four GST-NSP4$_{Wa}$ fusion proteins (one containing NSP4 full length, three C terminal truncated fragments). The truncated constructions were made taking the structural domains of the protein into consideration. Hydrophobic domains are indicated as H1 to H3. The toxigenic peptide (amino acids 114-135) is contained in the α-helix domain of the protein. The amino acids that mark the C terminal end of each construct are indicated by arrows.

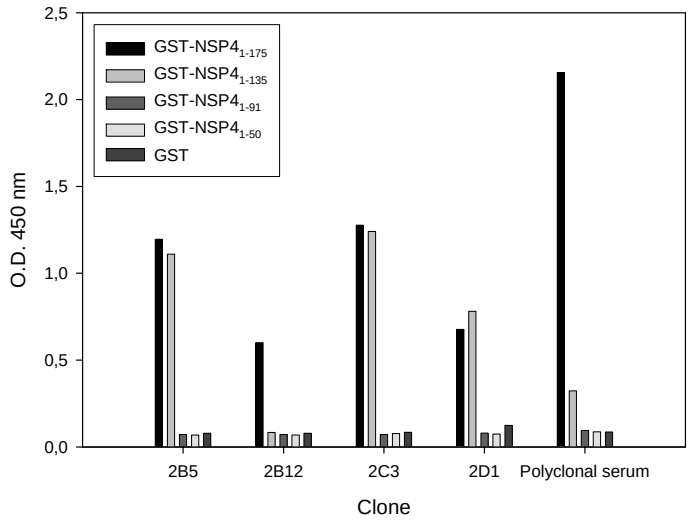

Figure 6. Reactivity of the different scFv clones and a polyclonal mouse serum obtained in mice against the four GST-NSP4 constructs (one full length, three truncated, Fig. 5) as detected by ELISA.

4. Discussion

In this work the phage display technique was successfully used to select several human scFv clones that specifically recognize the NSP4 protein. After partial molecular characterization, four different scFv against the NSP4 protein from the human rotavirus strain Wa were selected. The four clones differed in their CDR3 sequences, and Western blot analysis suggested that only one clone (2B12) recognized a conformational epitope in NSP4. Phage display technology has been used to perform antigenic analysis of the rotavirus VP7 protein (Huang et al., 2000), to map the domain in VP4 that binds to cellular receptors (Jolly et al., 2001) and to isolate human anti-VP4 and anti-VP7 monoclonal antibodies that neutralize human rotaviruses (Higo-Moriguchi et al., 2004). Here this technique has been used to obtain antibodies against the NSP4 protein. The binding region of individual scFv antibodies on NSP4 was mapped by using four different truncated forms of NSP4wa. Three of the clones mapped to the NSP4 region between amino acids 91 and 135, which includes the toxigenic peptide (NSP4114-135).

Table 2. Reactivity patterns of scFv antibodies directed against the NSP4 protein: recognized region of NSP4, binding of scFv to NSP4 on Western blot after SDS-PAGE and reactivity against NSP4 genotypes A and B in ELISA.

scFv clone	recognized fragment[a]	Western blot binding	$NSP4_{Wa}$ genotype B	$NSP4_{SA11}$ genotype A
2B5	aa 91-135	+	+	+
2B12	aa 135-175	-	+	+
2C3	aa 91-135	+	+	+
2D1	aa 91-135	+	+	-

[a]The region of NSP4 that is recognized by each clone is indicated in amino acid (aa) positions.

A recent antigenic analysis of the NSP4 of avian rotavirus strain PO-13 demonstrated four different antigenic sites (AS) (Borgan et al., 2003). Two of these sites are immunodominant and are placed in regions spanning amino acids 151 to 169 (AS I)

and amino acids 136 to 150 (AS II). Similarly, our results obtained with mouse polyclonal anti-NSP4 sera showed that the region between amino acids 135 and 175 of NSP4Wa protein is immunodominant. However, the phage display technique allowed us to generate scFvs antibodies specifically directed against a non-immunodominant region (amino acids 91 to 135). This can be explained because in this technique the immune system is not involved in the selection of the scFv. Therefore, phage display can be a valuable tool to isolate monoclonal reagents suitable to perform functional analyses on NSP4. Especially, scFv antibodies reactive with the toxigenic region (clones 2C3, 2B5 and 2D1) could be particularly useful to study toxicity mechanisms.

Passive and active immunizations against the NSP4 protein have protected mice from suffering diarrhea after both NSP4 and virulent rotavirus challenges (Ball et al., 1996; Yu and Langridge, 2001). Neutralizing experiments with scFv antibodies or Fab fragments against structural viral proteins obtained by the phage display technology have been successfully carried out with human rotaviruses (Higo-Moriguchi et al., 2004) as well as with measles virus (de Carvalho Nicacio et al., 2002). Moreover, scFv antibodies have the advantage that they can be cloned, manipulated and produced in different hosts (Robin et al., 2003). Therefore, the scFv antibodies specific for NSP4 can be expressed in different cellular hosts in order to perform studies on the NSP4 in vivo functions. Ongoing studies in our laboratory are trying to elucidate whether these scFv antibodies can be used to label NSP4 proteins and whether they can reduce in vivo the ability of NSP4 to produce diarrhea in mice. In addition, it will be necessary to evaluate whether they can block the increase in intracellular calcium levels, neutralize the reduction in transepithelial resistance (TER) in polarized cells induced by NSP4 and interfere with rotavirus replication (Tian et al., 1994; Ciarlet et al., 2001; Estes, 2003).

Although only NSP4 from two genotypes (A and B) of NSP4 have been tested in this study, we were able to obtain scFv clones that differentiated between the two NSP4

genotypes. Clone 2D1 only reacted against the protein region between the amino acids 91 to 135 of genotype B, while the rest of clones recognized both A and B genotypes. These different reactivities can be explained by the diversity of amino acid sequences in that particular region of the NSP4. Recent studies have shown that NSP4 from genotypes A and B are the most common genotypes of NSP4 in humans (Iturriza-Gomara et al., 2003). We consider that future studies on the specificity of the isolated scFv antibodies against a broader range of NSP4 proteins derived from strains of different genotypes will show whether these antibodies will be useful to establish a serotypic classification of NSP4 of human rotaviruses.

Acknowledgments

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CAPÍTULO 2B

Selection of single-chain antibodies against the VP8 subunit of rotavirus VP4 outer capsid protein and their expression in Lactobacillus casei*

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ABSTRACT

Single-chain antibodies (scFv) recognizing the VP8* fraction of rotavirus outer capsid and blocking rotavirus infection *in vitro* were isolated by phage display. Vectors for the extracellular expression in *Lactobacillus casei* of one of the scFv were constructed. *L. casei* was able to secrete active scFv to the growth medium, showing the potential of probiotic bacteria to be engineered to express molecules suitable for *in vivo* anti-rotavirus therapies.

Keywords: rotavirus, VP8*, single-chain antibodies, lactic acid bacteria

Running title: Phage antibodies against VP8*

Group A rotaviruses are the main cause of diarrhea in children worldwide and are estimated to be responsible for more than 800.000 deaths per year in children under five years of age (12). The incidence of rotavirus disease in developing countries as well as the lack of an effective vaccine supports the development of new and more effective antirotaviral strategies. Mucosal immunity against rotavirus infection is believed to rely mainly on the production of rotavirus-specific IgA antibodies at the intestinal mucosal surface (12, 17, 26). Moreover, lacteal IgG or monoclonal IgA against the VP8* portion of rotaviral VP4 outer capsid can protect newborn mice against rotavirus-induced diarrhea (5, 21, 22). This makes VP8* a good target for the design of antirotavirus therapies.

Lactic acid bacteria (LAB) are GRAS microorganism present in numerous food fermentations and are also normal constituents of the intestinal habitat. In addition, some strains of LAB exhibit probiotic properties. These characteristics have been exploited for their use as live vectors for the expression of different peptides and its delivery to mucosal surfaces in animal models. These include: antigens (16), interleukins (24), enzymes (3) and single chain antibodies (scFv) (1, 13). These last molecules are chimeric proteins consisting in a fusion of the V_H and V_L variable regions of immunoglobulins (19). Specific scFv can be isolated by the phage display technique after panning of phage scFv libraries on immobilized antigen (7, 9). scFv offer very interesting clinical perspectives and, although they may not be as powerful as natural immunoglobulins, many possible applications could be envisaged, since they can be cloned, manipulated and produced in microbial hosts (20). Two cases of successful therapeutical application by *in vivo* delivery of scFv in mucosae by LAB have been reported (1, 13). We decided to construct *Lactobacillus casei* strains expressing extracellular or cell wall attached anti-VP8* scFv, which might be useful to deliver passive immunity against rotavirus. The use of *L. casei* might be of particular interest, since some strains exhibit an intrinsic beneficial effect in the treatment of rotaviral diarrhea (11, 18)

Isolation of scFv against VP8*

The Griffin.1 phage display library (6) was used to select phage antibodies against purified VP8* from rotaviral SA11 strain. This library is a semisynthetic human scFv library composed by more than 10^9 independent clones carrying V_H and V_L immunoglobulin variable regions cloned into pHEN2 (8) to produce a scFv fused to the pIII protein of the M13 viral capsid. Several rounds of panning and selection of VP8*-binding phages was carried out as described (9) with VP8*-coated immunotubes (Polysorp, NUNC). Titers of eluted phages and their signals in VP8*-specific ELISA increased after each round, indicating the enrichment of VP8* specific phages (data not shown).

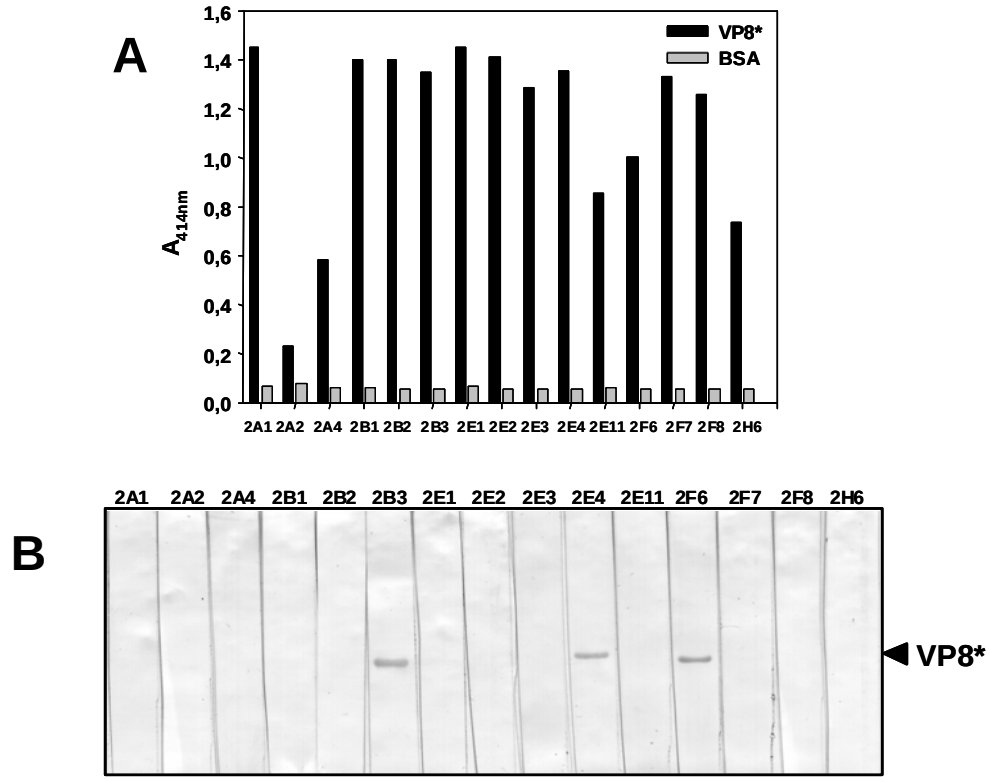

Figure 1 Isolation of anti-VP8* scFv by phage display. A) ELISA analysis of clones recognizing VP8* and isolated from rounds three and four. Bovine seroalbumine (BSA) was used as negative control. B) Western blot analysis of 50 ng of VP8* probed with 10^7 M13 phages/ml carrying anti-VP8* scFv::pIII fusions. The blots were developed with anti-M13-HRPO conjugated (Amersham).

Phages were rescued from several individual clones from the third and fourth rounds of selection and tested for their ability to bind VP8* by ELISA. From 96 assayed clones, 65 turned out to be positive, showing signals ranging from weak (A_{414} of 0.2) to strong positives (A_{414} > 1.5). Fifteen independent clones representing both weak and strong positives were randomly chosen and assayed in a second ELISA. As can be seen in Figure 1A, phages derived from all the clones gave a positive ELISA with VP8*, but not with the BSA negative control.

scFv characterization

Positive clones were assayed for recognition of VP8* by Western blot analysis using the whole M13 phages as an antibody reagent. Fifty nanograms of VP8* were subject to SDS-PAGE, electrotransferred to nitrocellulose membranes and probed with M13 phages (10^7 pfu/ml in PBS plus 2% skim milk). Blots were developed with an anti-M13 antibody conjugated to alkaline phosphatase (Amersham) and NBT/BCIP as substrate (Roche) (Figure 1B). Only clones 2B3, 2E4, and 2F6 proved to perform well in Western blot and were able to detect denatured VP8* transferred to nitrocellulose membranes, indicating the recognition of linear epitopes in VP8*. Clones were subject to PCR amplification of the scFv coding gene with oligonucleotides FOR_linkseq (5´-GCCACCTCCGCCTGAACC-3') and pHEN-SEQ (5'-CTATGCGGCCCCATTCA-3') and sequenced. Inspection of the complementarity determining region 3 (CDR3) of V_H revealed that the fifteen clones carried 8 different set of sequences (Table 1). Clones exhibited a high variability in their sequences, although four clones (2A1, 2B3, 2E3 and 2E11) had a biased amino acid composition in their CDR3, with preference for the basic amino acid arginine and for the presence of tryptophan. However, at this stage it cannot be stated whether scFv sharing homologous CDR3 are recognizing similar epitopes in VP8*. Surprisingly, one clone (2H6) was shown to be originated from a pHEN2 clone of the Griffin.1 library which did not receive the V_L region during cloning, and thus contained an in-frame fusion between the signal peptide carried by pHEN2,

the V_H region, the linker region and the pIII coding gene. Clones 2E4 and 2F6, which were functional in Western blot, were identical in sequence. The third positive clone in Western blot, 2B3, shared the same CDR3 sequence as clone 2E11, although they differed in their V_L set of sequences (Table 1), which might explain the inability of clone 2E11 to recognize denatured VP8* on a membrane.

Table 1. Amino acid sequences of V_H CDR3 and usage of V_H and V_L genes by the isolated scFv against VP8*

clone	V_H[a]	V_L[a]	CDR3 sequence
2A1, 2B1, 2B2, 2E1, 2E2, 2F7, 2F8	DP73	Vλ1	YRRNT
2E4, 2F6	DP49	Vλ3	SIPAP
2A2	DP31	Vλ3	QYSNVRL
2A4	DP12	Vλ3	GAMY
2B3	DP49	Vλ3	WRRAR
2E3	DP14	Vλ3	LWRTL
2E11	DP49	Vκ1	WRRAR
2H6	DP2	-	REQDALN

[a] Assignment to different V_H and V_L germ lines was done according to the V-BASE database of human immunoglobulin genes (25). The 2H6 clone did not contain a V_L segment.

In vitro inhibition of viral infection in MA104 cells

VP8* is implicated in the viral recognition of glycoproteins on the surface of mature enterocytes (4), and it is therefore involved in the first steps of viral entry (14, 16). VP8*-specific IgA monoclonal antibodies and mouse lacteal IgG antibodies are able to inhibit *in vitro* and *in vivo* rotavirus infections (5, 22), probably by preventing virus attachment to cells. We therefore tested the ability of our scFvs to inhibit rotavirus infection *in vitro*. A representative of each group of clones was chosen (clones 2A1, 2A2, 2A4, 2B3, 2E3, 2E4, 2E11 and 2H6). Phages displaying scFv::pIII fusions (10^9 pfu) were incubated with trypsin-activated SA11 rotavirus strain ($3X10^2$ pfu) in 50 μl of PBS and the mixture was used to infect MA104 cells monolayers grown in microtiter plates in MEM medium supplemented with 10% fetal bovine serum. The microtiter plates were centrifuged for 1 h at 1500 rpm and cells were washed with 100 μl of MEM. Two-hundred microliters of MEM containing 1 μg/ml trypsin without fetal bovine serum were added to each well and plates were incubated at 37ºC for 15 h. Then, cells were

washed with PBS and fixed for 10 min with 100 μl of acetone/methanol (1:1). Infection spots were developed with a goat anti-rotavirus serum (Chemicon) followed by an anti-goat IgG HRPO-conjugated and counted in a microscope. It could be observed that all the scFv-carrying phages were able to inhibit viral infection by 40 to 80 % compared to an M13 phage displaying an unrelated scFv or a control were no phage was added (Figure 2). According to Ruggeri *et al.* (21), a percentage of inhibition higher than 60 % can be considered indicative of a blocking antibody.

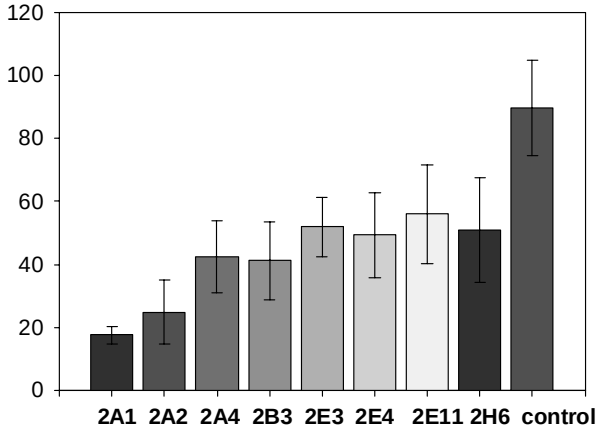

Figure 2. *In vitro* inhibition of SA11 virus infection in MA104 cells. Phages carrying scFv::pIII fusions were incubated with SA11 virus and the mixture was used to infect MA104 cells. SA11 proliferation was visualized by an anti-rotavirus goat serum and an anti-goat IgG HRPO-conjugated. The results are shown as percentage of infection spots relative to an experiment where no phage was added. The control represents a phage expressing an unrelated scFv.

Expression of scFv in *Lactobacillus casei*

One of the scFv giving a high SA11 strain blocking activity was chosen (2A1 clone) and its gene cloned in the LAB expression vector pRo266. This vector was obtained by replacing the *usp*45 secretion and *spaX* cell wall-anchoring signals in pT1NX (23) by the sequence for the signal peptide and cell wall anchor regions from *L. casei* cell wall proteinase, PrtP (10). Briefly, the gene fragment from the PrtP cell wall anchor signal (PrtPAnch) was amplified by PCR with the Expand High Fidelity PCR kit (Roche) and oligonucleotides 5'-CGAGTGGATCCAAGGTACTTGA-3' and 5'-ATGTTACAGCCATCGGTACCGCA-3' and *L. casei* chromosomal DNA as template

(restriction sites introduced in the oligonucleotides to facilitate cloning are underlined). The PCR product obtained was digested with *Bam*HI and cloned into pT1NX digested with *Bam*HI-*Spe*I(made blunt with the klenow enzyme). The resulting plasmid was digested with *Bam*HI and *Bgl*II(blunt) and ligated to the PCR product encoding the PrtP secretion signal (ssPrtP) obtained with oligonucleotides 5'-GGTTCTAGAACTTTTGGG-3' and 5'-ATGAGGATCCGTCGCCGGCCGAGATAGCCGCCTT-3' and digested with *Bam*HI, resulting in pRo266. The fragment encoding 2A1 scFv was amplified by PCR with oligonucleotides SCFV1 (5'-GCGGCCGGCCCGGCCATGC-3') and Fdseq1 (5´-GAATTTTCTGTATGAGG-3'). Then, it was digested with *Ngo*MIV and cloned into pRo266 digested with *Ngo*MIV/*Nco*I(blunt), yielding pScFv3, which contained a ssPrtP::scFv fusion. Plasmid pScFv4, carrying a ssPrtP::scFv::PrtPAnch fusion was constructed by cloning the 2A1 scFv coding region, amplified with oligonucleotides SCFV1 and SCFV3 (5'-CTGCGGCCCCATTCAGATCC-3') and digested with *Ngo*MIV, into pRo266 digested with *Ngo*MIV/*Bam*HI(blunt). Ligation mixtures were directly used to transform *Lactococcus lactis* MG1363 and sequencing of the corresponding plasmids was carried out to verify the correct sequences. Transformation of *L. casei* with the anchoring vector pScFv4 was very inefficient and this plasmid showed structural instability in this host (not shown). *L. casei* cells harboring pScFv3 were grown in 15 ml MRS medium for three hours and then resuspended in 15 ml of M9 medium supplemented with 0.5 % glucose, 0.1 % triptone, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 5 μg/ml erythromycin, buffered with 50 mM sodium carbonate pH 7.4 and grown for a further 4 hours. Cell supernatants and crude extracts were prepared as described (2). Samples were resolved by SDS-PAGE and probed in western blot with an anti-c-*myc* tag monoclonal antibody (1 μg/ml, Roche), which recognizes the C-terminal *myc* tag present in the scFv (8), followed by incubation with anti-mouse IgG conjugated to alkaline phosphatase and using BCIP/NBT (Roche) as the substrate. As can be seen in Figure 3A, cells were able to secrete mature scFv to the growth medium, although approximately one half of the protein remained intracellular, as the unprocessed form

(Figure 3A). The scFv secreted by *L. casei* cells was 50-fold concentrated from the growth medium with an Amicon Ultra centrifugal filter device (10 kDa molecular weight cut-off, Millipore) and used in an ELISA to VP8*. scFv binding was detected with the anti-c-myc monoclonal antibody (4 μg/ml) and a 1:2000 dilution of anti-mouse IgG alkaline phosphatase-conjugated (Roche). As shown in Figure 3B, 2A1 scFv was able to recognize SA11 VP8*. This indicated that the secreted scFv had a correct folding that would retain its biological activity and demonstrated the potential of *L. casei* to be engineered to express such antibodies. Our future work will be aimed to the optimization of scFv-producing strains and their test in an *in vivo* rotavirus infection mice model. In particular, new constructs designed to display the scFv on *L. casei* surface would be necessary, as cells might function as multibinding reagents in VP8* recognition, increasing avidity.

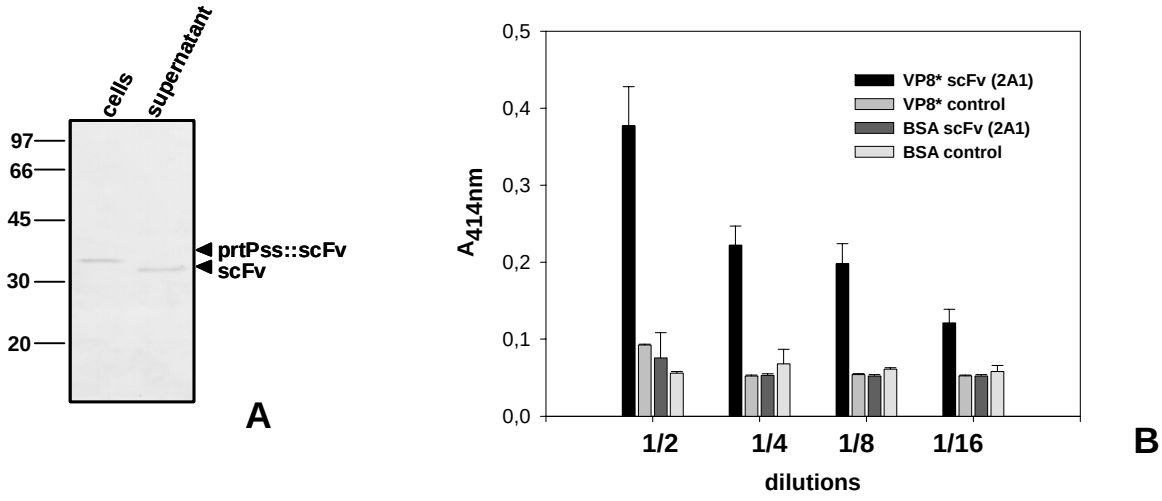

Figure 3 Expression and functionality of 2A1 scFv in *Lactobacillus casei*. A) Western blot of cells extract and supernatant from *L. casei* [pScFv3] equivalent to 1 ml of culture, probed with an anti-c-*myc* tag which recognized the tagged scFv. Molecular weight markers are indicated in KDa. B) Functionality of 2A1 scFv secreted by *L. casei* as determined by ELISA. Culture supernatants of *L. casei* secreting 2A1 scFv were 50-fold concentrated and several dilutions were assayed for VP8* recognition by ELISA. The control represents supernatants of an *L. casei* strain carrying the cloning vector pRo266. BSA was used as negative control.

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CAPÍTULO 3

Humoral immune response to rotavirus NSP4 enterotoxin in Spanish children

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ABSTRACT

The rotavirus nonstructural protein 4 (NSP4) has been shown to play a crucial role in rotavirus-induced diarrhoea, acting as a viral enterotoxin. It has also been demonstrated that antibody to NSP4 can reduce the severity of rotavirus-induced diarrhoea in newborn mice. Two recombinant baculoviruses expressing the NSP4 protein from the SA11 and Wa rotavirus strains, genotypes A and B respectively, were used to produce and purify these glycoproteins, which were applied as antigen in an enzyme-linked immunosorbent assay (ELISA) to analyse the specific antibody response to NSP4 in human sera. Serum samples from 30 children convalescing from a rotavirus infection, from 54 healthy children under 5-years-old and from 49 adults were tested to determine the presence of antibodies to the viral enterotoxin and to rotavirus particles. Seventy percent of the sera from rotavirus-infected children showed an IgG antibody response to either one or both NSP4 proteins used in this study, although the response was weak. However, IgG antibodies towards either one or both NSP4 proteins were only detected in 26% of the non-convalescent healthy children and in only 18% of the adults. No serum IgA antibodies towards NSP4 were found in this study. IgG antibody recognition of the NSP4 protein from the SA11 and Wa rotavirus strains was not always heterotypic.

Shortened title: Antibody response to rotavirus NSP4 protein

Key words: rotavirus gastroenteritis, nonstructural protein 4, enterotoxin, antibodies.

INTRODUCTION

Rotaviruses (RV) are the main etiologic agents of viral gastroenteritis in infants and young children worldwide, producing high childhood mortality in developing countries (Parashar et al., 2003b). The importance of the rotavirus diarrhoea has encouraged several research groups to develop rotavirus vaccines. The vaccine candidates include the bovine rotavirus UK-based tetravalent vaccine, the rhesus rotavirus-based tetravalent vaccine and the bovine rotavirus NCDV-RIT4234 vaccine (Bern and Glass, 1994, Kapikian et al., 2001, Parashar et al., 2003a). At present two different rotavirus vaccines are in a late-stage of development; these vaccines are a monovalent human rotavirus (P1A(8), G1) vaccine and a pentavalent bovine-human reassortant vaccine (Barnes et al., 2002, Bern and Glass, 1994, Offit, 2002, Richardson et al., 1993). The importance of structural proteins, such as VP7 and VP4, which are able to induce neutralising antibodies has been extensively studied (Conner et al., 1993, Contreras et al., 1995, Coulson, 1993, Crawford et al., 1999, Gorrell and Bishop, 1997, Herrmann et al., 1999). However, much controversy exists as to whether these antibodies are directly involved in protection against rotavirus-induced diarrhoea (Jiang et al., 2002).

The rotavirus nonstructural glycoprotein NSP4, encoded by rotavirus gene 10, is an intracellular receptor that mediates the acquisition of a transient membrane envelope as subviral particles are translocated across the endoplasmic reticulum (ER) membrane (Estes, 2001). Purified NSP4 or a peptide corresponding to NSP4 amino acids 114-135 induce diarrhoea in young mice, suggesting a role of NSP4 in rotavirus pathogenesis (Ball et al., 1996, Horie et al., 1999). NSP4 and NSP4 (114-135) peptide increase chloride secretion by a calcium-dependent signalling pathway acting as a viral enterotoxin (Ball et al., 1996). A specific fragment of NSP4 (amino acids 112 to 175) is secreted via a nonclassical pathway early after cellular infection (Zhang et al., 2000), and NSP4 or this fragment exogenously applied to cells causes an increase in intracellular calcium via the phospholipase C-inositol 1,3,5-triphosphate cascade that

releases Ca^{2+} from the ER (Berkova et al., 2003, Dong et al., 1997, Morris et al., 1999, Tian et al., 1995, Tian et al., 1994). In contrast, the increase in intracellular calcium induced by intracellularly expressed NSP4 is independent of PLC stimulation (Tian et al., 1995). NSP4 also causes membrane destabilisation associated with a region of the protein located within residues 48-91, which includes a potential cationic amphipatic helix (Browne et al., 2000, Huang et al., 2004, Huang et al., 2001, Tian et al., 1996). Recent studies indicate that the enterotoxigenic peptide NSP4 114-135 also inhibits the Na+-D-glucose symporter (SGLT1) in the rabbit intestinal brush border membrane. This inhibition implicates an interference of water reabsorption, which may also play a role in the pathogenesis of rotavirus diarrhoea (Halaihel et al., 2000).

Recent genetic studies revealed the presence of six distinct alleles, genotypes or genetic groups (from A to F) including animal and human strains (Cao et al., 1999, Ciarlet et al., 2000, Cunliffe et al., 1997, Kirkwood and Palombo, 1997, Mori et al., 2002). Genotype A includes human, bovine and simian strains; NSP4 genotype B includes human and porcine strains, genotype C includes human, simian, canine and feline strains, genotype D is composed of murine strains, while genotypes E and F include avian NSP4 proteins (Ciarlet et al., 2000, Mori et al., 2002).

It is important to investigate whether the antibodies against NSP4 are type-specific or cross-reactive. The immunisation against the three genotypes characterised so far in human rotaviruses (A, B and C) would be necessary to achieve complete protection if the immune response is homotypic. However, very few studies have been carried out to elucidate the immunologic determinants in the NSP4 protein. Recently two immunodominant regions have been described in the NSP4 of group A avian rotavirus PO-13 (P(17), G7, NSP4 genotype E). The antigenic site I (AS I) (amino acids 151 to 169) and the antigenic site II (AS II) (amino acids 136 to 150) are the two immunodominant regions and are located in the cytoplasmatic tail (Borgan et al., 2003). Two of these genotypes have been shown to be prevalent in human infections.

NSP4 proteins corresponding to genotypes A and B have been detected in a recent study in almost all the rotavirus strains infecting humans (Iturriza-Gomara et al., 2003).

Although several studies have analysed the importance of the immune response against NSP4, the role that both humoral and cell-mediated immune responses to NSP4 may play in the protection against rotavirus infection is still unknown. NSP4 has been shown to induce both humoral and cell-mediated immune responses in humans (Johansen et al., 1999, Ray et al., 2003, Yuan et al., 2004). Passive and active immunisations against the NSP4 protein have protected mice from suffering diarrhoea after both NSP4 and virulent rotavirus challenges (Ball et al., 1996, Yu and Langridge, 2001). It has been predicted that antibody to NSP4 should reduce rotavirus-induced diarrhoea, inducing a similar protection to that elicited by antibodies to bacterial enterotoxins against bacteria-induced diarrhoea (Estes, 2003).

In this study, two recombinant baculoviruses expressing the NSP4 rotavirus proteins from the simian strain SA11 (genotype A) and from the human rotavirus strain Wa (genotype B) were employed. The recombinant proteins were purified and used to coat microtiter ELISA plates to determine antibodies to NSP4 in human sera. Sera from rotavirus-infected children, uninfected children and adults were tested to analyse the prevalence and the titers of both IgG and IgA antibodies against NSP4. The results indicate a high prevalence of anti-NSP4 antibodies in infected children though at low or very low titer levels. A low prevalence of anti-NSP4 antibodies was found among non-convalescent healthy children and adults.

MATERIALS AND METHODS

Viruses and cells

Simian rotavirus strain SA11 (P5B(2), G3) and Wa (P1A(8), G1) were obtained from the American Type Culture Collection (ATCC ref. 899-VR and 2018-VR, respectively). Both strains were propagated in confluent MA104 cells in the presence of 1 µg/ml

trypsin (type IX, Sigma Chemical Co., St. Louis, MO). *Spodoptera frugiperda* Sf9 insect cells (ATCC ref. CRL-1711) were grown in Grace's medium (Gibco Invitrogen Corp., Paisley, Scotland, UK) supplemented with 10% foetal bovine serum.

Expression and purification of NSP4 proteins in insect cells

Recombinant baculoviruses expressing the NSP4s proteins from SA11 and Wa strains were used to produce and purify the recombinant NSP4s from genotypes A and B as previously described (Rodriguez-Diaz et al., 2003). Briefly, Sf9 cells were infected with the recombinant baculoviruses at a multiplicity of infection (m.o.i.) of 10. After 48 h of incubation, cells were harvested by centrifugation at 500 g for 5 min and resuspended in buffer A (phosphate buffer 50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 10 mM imidazole) containing 1% Triton X-100 and 0.1% protease inhibitor cocktail (Sigma, St. Louis, MO) for lysis. The lysates were diluted in the same volume of buffer A (1:1) and centrifuged at 22.000 g at 4ºC for 5 min. Clarified lysates were incubated with 1 ml Ni-NTA (Qiagen) for 30 min at 4ºC with shaking. The resin was then washed with 3 ml buffer A, 20 mM imidazole and with 3 ml of buffer A, 50 mM imidazole. The proteins were eluted with 3 ml buffer A, 250 mM imidazole, and 0.5 ml fractions were collected. Proteins were quantified by the Bradford method, analysed by SDS-PAGE and Coomasie blue stained.

Subjects and serum samples

Sera were collected from 30 children less than 5 years of age convalescent of an acute rotavirus infection, 15 to 20 days after the onset of diarrhoea; from 54 non-convalescent healthy children under 5-years-old and from 49 adults 20-35 years of age.

Detection of serum IgG antibodies to Wa and SA11 rotavirus by ELISA

Titers of serum IgG to rotavirus strains Wa and SA11 were determined by ELISA as described (Colomina et al., 1998). Briefly, 96-well microtiter plates were coated overnight at 4ºC with CsCl density gradient-purified rotavirus particles (SA11 or Wa strains) previously treated with 1.5 M sodium thiocyanate and diluted in carbonate-bicarbonate buffer (pH 9.6) at a final concentration of 2 µg/ml. Serum samples were serially diluted, starting at 1/100, in PBS-Tween (PBS-T) containing 1% bovine serum albumin (PBS-T-BSA). 100 µl were added and incubated for 2 h at 37ºC, and the plates were washed three times with PBS-T. Horseradish peroxidase-conjugated anti-human IgG goat antibody (Sigma Immunochemicals, St. Louis, MO) diluted 1/2000 in PBS-T-BSA was added. The plates were incubated 2 h at 37ºC, and washed three times with PBS-T. Reactions were developed by adding o-phenylendiamine (Sigma Immunochemicals) in citrate-phosphate buffer (pH 5) with 0.1% H_2O_2. The reaction was stopped with 3M H_2SO_4. Plates were read at 492 nm in a spectrophotometer. As a control for specificity, wells were coated with the same amount of protein from MA104 mock-infected cells. A sample was considered positive when its OD was three times higher than the absorbance value of the same sample in the specificity control. The titer of each sample was considered the inverse of the last dilution showing a positive reaction.

Detection of serum antibodies to $NSP4_{Wa}$ and $NSP4_{SA11}$ antigens by ELISA

ELISA tests were performed with purified NSP4 proteins from the SA11 and Wa rotavirus strains produced by recombinant baculoviruses to detect antibodies to $NSP4_{Wa}$ and $NSP4_{SA11}$ following a similar procedure to that described above. Mock-infected Sf9 cells were processed in the same way as the baculovirus-infected Sf9 cells producing the recombinant proteins, and were used as specificity controls. Quantitation of IgA/IgG was carried out by assaying each serum sample at different dilutions, starting at 1/25 for both Ig isotypes. Horseradish peroxidase-conjugated goat anti-

human IgA (α-chain specific) and anti-human IgG antibodies (Sigma Immunochemicals, St. Louis, MO) were used to detect human IgA and IgG antibodies, respectively. A test sample was considered positive when the OD was three times higher than the absorbance value of the same sample in the specificity control. As in the ELISA against the rotavirus particles, the titer of each sample was considered the inverse of the last dilution that showed a positive result.

Statistical analyses

Comparisons between the mean titers of antibodies to NSP4 and to whole rotavirus obtained in the three different groups (convalescent children, non-convalescent healthy children and adults) were carried out with the *t-student* analysis. Categorical variables were compared by χ^2 test. $P<0,05$ was considered significant. The SPSS software (Lead Technologies, Inc.) was used to perform these analyses.

RESULTS

Antibodies to NSP4 in rotavirus-infected children

Seventy per cent of convalescent serum samples collected from rotavirus-infected children were found to contain IgG antibodies against at least one of the two NSP4 proteins used in this study (Table I). Twelve sera (40 %) recognised both $NSP4_{Wa}$ and $NSP4_{SA11}$ proteins. This result indicates a heterotypic recognition by the antibodies to NSP4 in these 12 samples or the event of previous infections with rotavirus carrying NSP4s from the two genotypes used in this study. Five sera (16.6 %) recognised only $NSP4_{SA11}$ (genotype A) but failed to recognise $NSP4_{Wa}$ (genotype B), and 4 (13.3 %) reacted only to $NSP4_{Wa}$ (Table I). Statistical analyses of these data demonstrated a significant difference in the recognition between $NSP4_{Wa}$ and $NSP4_{SA11}$ (P = 0.03), suggesting that recognition of both NSP4 proteins is not heterotypic. Comparison of the

mean values of IgG antibody titers in all the convalescent serum samples was established with the titers found in the samples from the uninfected children and the adults (Fig. 1A). No IgA antibodies to either of the recombinant NSP4 proteins were detected.

Table 1. Percentage of serum samples from convalescent children of an acute rotavirus infection, non-convalescent children and adults containing IgG antibodies to $NSP4_{Wa}$ (genotype B), $NSP4_{SA11}$ (genotype A) or to both NSP4 proteins.

	Convalescent children	**Uninfected children**	*Adults*
$NSP4_{Wa}$/$NSP4_{SA11}$	12 (40%)	7 (12.9%)	3 (6.1%)
$NSP4_{Wa}$	4 (13.3%)	4 (7.4%)	6 (12.2%)
$NSP4_{SA11}$	5 (16.6%)	3 (5.5%)	0 (0%)
Negative	9 (30%)	40 (74%)	40 (81.6 %)
Total	30	54	49

Antibodies to NSP4 in uninfected children and adults

Fourteen serum samples (26 %) from the uninfected children recognised at least one of the NSP4 proteins (Table 1). The distribution of the positive samples was as follows: 7 sera (12.9 %) reacted to both proteins, 4 samples (7.4 %) were positive against $NSP4_{Wa}$ and 3 samples (5.5 %) against $NSP4_{SA11}$. Nine serum samples (18.4 %) from the adults were found positive for antibodies against at least one of the NSP4s proteins: 3 of them (6.1 %) recognised the NSP4 from both SA11 and Wa strains and 6 (12.3 %) were only reactive towards the NSP4 from the human Wa strain.

Antibodies to rotavirus particles

All the serum samples showed high titers of IgG antibodies against complete rotavirus particles, both viral strains Wa and SA11, indicating that all the rotavirus-infected children had seroconverted. The mean titers were compared among the three groups (Fig. 1B).

A high prevalence of serum IgG antibodies to whole rotavirus was found both in the uninfected children and in the adults; this seroprevalence was higher than 80% in the non-convalescent children indicating previous rotavirus infection(s). The percentage of sera from the adults reacting against rotavirus particles was higher than 90%. All the samples recognising the NSP4 protein also reacted with the rotaviral particles.

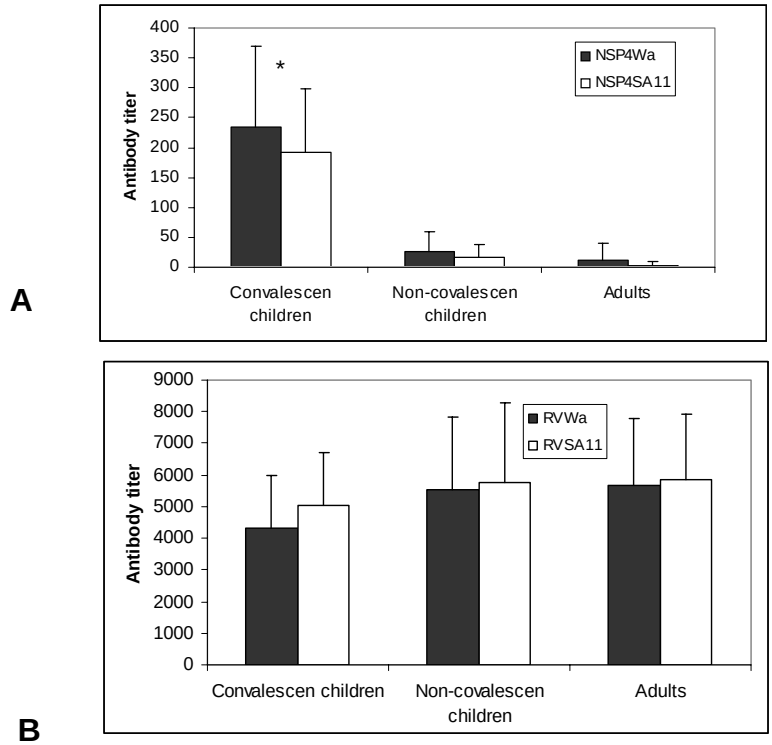

Figure 1. IgG antibody mean titers to NSP4 proteins (panel A) and to whole rotavirus particles (panel B) in convalescent children, non-convalescent children and adults. Panel A shows the average antibody titers against both NSP4 proteins NSP4Wa and NSP4SA11.The asterisks show the presence of significative differences. Panel B represents the average titers of the sera recognising the complete rotavirus particles of strains Wa and SA11. The error bars are indicating the standard deviation (SD).

Comparison of the antibody titers between children with rotavirus diarrhoea, healthy children and adults

Comparing the mean values of serum IgG antibody titers against the NSP4 proteins between the three groups showed that the infected children presented higher titer levels towards the NSP4 from the Wa or SA11 strains than the adults or the uninfected children (P<0.001). The comparison of the mean titers obtained from the uninfected

children and the adults did not show significant differences (*P*> 0.05). When the comparison was established between the serum titers obtained against the full rotavirus particles no differences were detected (*P*>0.05).

The number of positive and negative serum samples against both NSP4 genotypes A and B were also compared between the three groups. The infected children presented a higher percentage of positive samples to either of the NSP4s proteins than the uninfected children or the adults (*P*<0.001). No statistical differences were found between the uninfected children and adults (*P*>0.05). The number of positive serum samples against rotavirus particles did not differ among the three groups (*P*>0.05).

DISCUSSION

This study describes the antibody response to the NSP4 protein in children convalescing from acute rotavirus diarrhoea, in non-convalescent healthy children and in healthy adults. In the convalescent children we found a very high prevalence of IgG antibodies to the genotypes of NSP4 that are more common in human infections (Iturriza-Gomara et al., 2003). We have found that human serum antibodies can discriminate between NSP4 genotype A and NSP4 genotype B in naturally infected children. This result does not accord with those reported from rotavirus-infected children in India by (Ray et al., 2003), who postulated a cross-reactivity of IgG antibodies to NSP4 genotypes A, B and C. Our results are in accordance with those obtained by Yuan et al. (2004), who detected both homotypic and heterotypic responses to NSP4 in children vaccinated against rotavirus. We found that in naturally infected children the IgG titer levels against the NSP4 proteins are low or very low, as previously described by Johansen et al. (1999) as opposed to the high titers described by Ray et al. (2003). In our study, no IgA response to the NSP4 proteins was detected in the convalescent sera. By contrast, Ray et al. (2003) found by ELISA IgA

seroconversions to NSP4 in children with acute rotavirus gastroenteritis. The main difference between these IgA ELISA protocols is that Ray et al. used biotin-conjugated anti-human IgA and horseradish peroxidase-conjugated avidin-biotin, which may have increased the sensitivity of the technique. However, similar to our results, Johansen et al. (1999) did not detect either IgA antibodies to NSP4.

The results in convalescent children show that the nonstructural protein NSP4 elicits IgG antibodies that can play a role in the protection against the enterotoxic function of this protein, as demonstrated in mice (Ball et al., 1996). It is important to investigate whether the antibodies against NSP4 are type-specific or cross-reactive. The fact that the antibodies to NSP4 can discriminate between both genotypes A and B enables us to develop serotyping procedures to determine the NSP4 genotype of rotavirus strains. With this purpose we have developed single-chain variable fragment (scFv) antibodies against NSP4 and some of these antibodies discriminate both NSP4 genotypes A and B (Rodriguez-Diaz et al., 2004).

Antibodies are generally considered to be a proxy for protection against rotavirus reinfection because they are a marker for past exposure, although it remains unclear which viral proteins might induce a protective antibody response (Jiang et al., 2002). In this study we found that a very high proportion of non-convalescent healthy children and adults have suffered previous rotavirus infections, due to the high prevalence of antibodies to whole rotaviral particles. Comparison of the average titers of IgG antibodies against the NSP4 proteins in these two groups did not showed statistical differences. Moreover, we found a low prevalence of IgG antibodies against the NSP4 proteins in both groups, in spite of having previously experienced rotavirus infections. This result does not accord with those obtained among Swedish and Nicaraguan adults (Johansen et al., 1999), in which the prevalence of IgG antibodies against NSP4 genotype A is high in the adult population. However, the antibody titers were always low or very low, as observed in our study.

We found that 74% percent of the healthy children and 81.6% of the adults have no antibodies against NSP4. Thus, one can hypothesize that reinfection with rotavirus would probably provoke diarrhoea in them, an assumption that could be mistaken. It is well known that in humans rotavirus infections happen throughout life but the resulting disease is mild and often asymptomatic (Bishop, 1996). Besides, NSP4 activity as an enterotoxin in the murine model shows an age dependent pattern (Ball et al., 1996), inducing diarrhoea only during the first 15 days of age.

In conclusion, we show that the NSP4 protein can elicit an IgG antibody response in humans after natural rotavirus infections and that the recognition of different NSP4s by these antibodies does not seem to be always heterotypic. We have also found that the prevalence of antibodies against NSP4 is very low among non-convalescent healthy children below 5 years of age, as well as in adults supposed to be protected from rotavirus disease upon reinfection.

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CAPÍTULO 4

Nitric oxide production during clinical and experimental infection with rotavirus

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ABSTRACT

The pathophysiological mechanisms behind rotavirus-induced diarrhoea still remain incomplete. Current views suggest that the non-structural protein 4 (NSP4) of rotavirus and the enteric nervous system (ENS) participate in water secretion and diarrhoea. In the present work the role of nitric oxide (NO) in rotavirus infection and disease have been studied in vitro, mice and humans. Incubation of human intestinal epithelial cells (HT-29) with purified NSP4 but not with infectious virus produced NO_2/NO_3 accumulation in the incubation media. The NSP4-induced release of NO metabolites occurred within the first minutes after the addition of the toxin. Mice infected with murine rotavirus (strain EDIM) accumulated NO2/NO3 in their urines; newborn pups (n=10) at the onset for diarrhoea and adult mice (n=10) at the onset for virus shedding.

Following rotavirus infection, iNOS mRNA was upregulated in ileum, but not in duodenum or jejunum of newborn pups within five days post infection. A prospective clinical study including 46 children with acute rotavirus infection and 19 age-matched controls concluded that rotavirus infection stimulates a strong NO metabolites production ($p<0.001$) during the course of the disease. Altogether these observations identify NO as an important mediator of host responses during rotavirus infection.

Key words: rotavirus, NSP4, enterotoxin, nitric oxide.

Running Title: Rotavirus stimulates nitric oxide production.

INTRODUCTION

Rotaviruses are the main etiologic agents of viral gastroenteritis in infants and young children worldwide, and are responsible for significant mortality in developing countries and high morbidity in developed countries (27). Rotavirus infects the mature enterocytes located in the upper half of intestinal villi that ultimetly leads to cell death. A striking observation in both animals and humans is that only a few percent of the mature villous epithelial cells and no crypts seem to be infected (24, 25, 28, 33), all suggesting that there is no absolute correlation between histological lesions and clinical symptoms. Mechanisms proposed to explain the rotavirus induced fluid and electrolyte secretion includes villous ischemia (25, 26), the participation of the NSP4 enterotoxin (4) and activation of the enteric nervous system (18, 19). Direct evidence for participation of the enteric nervous system (ENS) in rotavirus diarrhoea in mice and humans has been reported (6, 14, 17, 32). While these studies indicated that a significant part of the reported net fluid secretion is mediated by the secromotor reflex, including serotonin, acetylcholine and VIP (6, 14, 17, 32), the specific details of how the secretor nervous reflex is activated are currently unknown. It has been hypothesised that rotavirus or its toxin may influence enteroendocrine cells to release

peptides/amines which, in turn, may activate nervous dendrites located just underneath the intestinal epithelium. Another proposal infers that enterocytes in response to luminal rotavirus release nitric oxide (NO), which, directly or indirectly may stimulate enteric afferents. None of the two hypotheses has been examined and in this study we have investigated the possible role of NO (19, 23, 28).

NO is a major secretory product of mammalian cells that participates in host defence, homeostatic and development functions. Activation of the immune system is associated with an increase in macrophage NO production. NO is also a neurotransmitter in the peripheral nervous system controlling e.g. smooth muscles in the gastrointestinal tract and in the vasculature (9, 10). The enzyme NO synthesase (NOS) exist in two main forms, a constitutive form (cNOS) and an isoform that is inducible (iNOS). The isoforms of the cNOS were named after the tissue in which they were found, neuronal NOS (nNOS) and endothelial NOS (eNOS) (2). The activity of both cNOS is regulated by the intracellular calcium concentration influenced by physiological stimuli (12, 21). iNOS, on the other hand, is calcium independent (34). In the immunological system iNOS is induced by cytokines at the transcriptional level primarily in macrophages, where NO is produced in high concentrations for long periods of time (43).

In the mid 1990's, the non-structural protein 4 (NSP4) of rotavirus and a 22 amino acid synthetic peptide of the protein ($NSP4_{114-135}$) were reported to induce an age-dependent diarrhoea in mice in the absence of histological alterations (4). Further studies showed that NSP4 and the $NSP4_{114-135}$ peptide exogenously applied to mammalian cells caused an increase in intracellular calcium. This calcium mobilisation from the endoplasmic reticulum (ER) was found to occur after the activation of the C phospholipase (7, 40). These observations in conjunction with the calcium dependence of cNOS synthesis and the possibility that NO may activate a secretory nervous reflex prompted the present study.

In the present work, we show that human intestinal epithelial cells (HT-29) responded with release of NO metabolites from the NSP4 toxin of rotavirus but not from productive rotavirus infection possibly through the calcium-dependent cNOS. Furthermore, animal experiments revealed that newborn mice responded with release of NO derivatives that correlated with onset of diarrhoea and adult mice with onset of virus shedding. Furthermore, iNOS mRNA from ileum, but not jejunum and duodenum was upregulated within five days post-infection in newborn mice. A most novel observation was that children with severe rotavirus disease released significant NO derivatives, all suggesting that NO play an important role during rotavirus infection.

MATERIALS AND METHODS

Expression and purification of NSP4 protein in insect cells

Recombinant baculovirus expressing the NSP4 protein from SA11 (G3, P5B[2]) strain was used to produce and purify the recombinant NSP4 as previously described (30). Briefly, Sf9 cells were infected with the recombinant baculovirus at a multiplicity of infection (m.o.i.) of 10. After 48 h of incubation, cells were harvested by centrifugation at 500 X g for 5 min and resuspended in buffer A (phosphate buffer 50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 10 mM imidazole) containing 1% Triton X-100 and 0.1% protease inhibitor cocktail (Sigma) for lysis. The lysates were then diluted in the same volume of buffer A (1:1) and centrifuged at 22.000 X g at 4ºC for 5 min. Clarified lysates were incubated with 1 ml Ni-NTA (Qiagen) for 30 min at 4ºC with shaking. The resin was then washed with 3 ml buffer A, containing 20 mM imidazole and with 3 ml of buffer A, containing 50 mM imidazole. The protein was finally eluted with 3 ml buffer A, containing 250 mM imidazole, in 0.5 ml fractions. The protein was extensively dialysed against PBS, quantified by the Bradford method and analysed by SDS-PAGE.

NSP4 inoculation and rotavirus infection in HT-29 cells

HT-29 human intestinal epithelial cells were placed in 24 well plates (Nunc) and incubated at 37°C 5% CO_2, with 1 ml of media in each well (MEM Eagle Medium 25mM HEPES 10% foetal calf serum). After the cells reached confluence the monolayers were washed 3 times with Dulbecco's modified Eagle medium (DMEM) without phenol red and different concentrations of purified NSP4 were added to the cells (from 1 nM to 500 nM) in 400 μl of total serum free DMEM without phenol red. Several wells were used as negative control by adding Sf9 antigen from non infected Sf9 cells (30) in the same volume of media. The cells were incubated with the toxin from 5 minutes to 48 hours at 37°C 5% CO_2. After the incubation the supernatants were carefully recovered and stored at -80°C.

In other experiments HT-29 cells growing in 24 well plates as described above were infected with trypsin-activated rotavirus SA11 strain. Prior to infection (m.o.i. 0.01-10), monolayers were washed 3 times with serum free D-MEM without phenol red. Virus was incubated for 1 h at 37ºC 5%CO_2 and the monolayers were subsequently washed and 400 μl D-MEM without phenol red were added to each well for 24, 48 and 72 hours. After each incubation the supernatants were carefully recovered and stored at -80ºC.

Rotavirus infection in mice

Rotavirus naïve adult and newborn BALB/c mice were infected with the murine rotavirus strain EDIM (G3 P10 [16]) as described previously (14) .Two groups of 10 adult BALB/ mice each group were orally administered at the age of 8 weeks, the first group with 10 μl of PBS containing 5 X 10^4 fluorescent forming units (FFUs) of EDIM rotavirus and the second group was mock infected. Two groups of 10 newborn Balb/c

mice (7 days old) were treated as the adult mice. All the mice were followed for two weeks after infection. Urine and stool samples were collected daily and stored at -80ºC.

Human samples

A total number of 46 urine and stool samples were collected during the months of February to May of 2004 in the hospitals of Leon and Chilandega in Nicaragua from children under 2 years of age suffering from severe rotavirus diarrhoea. The study included children of mothers who agreed to participate after information about the study. The urine and stool samples were collected the next day after the hospitalization of the children. Clinical data obtained from an attending paediatrician included temperature, presence or absence of vomiting and hydratation status, evaluated according to standarized scheme of signs and symptoms. Three different states of dehydration were assigned, mild, moderate and severe (8). The samples were stored at –20º C until further use. Also 19 samples of urine and stools were collected from healthy age-matched children from the same geographic localisation.

Detection of rotavirus by ELISA

Detection of rotavirus in the supernatants of the infected cells or in the stool specimens from mice and humans were performed by ELISA as previously described (37).

Nitrite/Nitrate measurements

To analyze the concentration of the stable oxidation products of NO in the supernatants and the urine samples, the total concentration of nitrate and nitrite (NO_2/NO_3) was calculated following the protocol designed by Verdon et al (42). Briefly 50 μl of the cell supernatants or from a dilution 1/50 of the urine samples were placed in each well of a 96 well plate. Then 10 μl of NADPH 10 μM were added (final concentration 1 μM) and 40 μl of a master mix (100 μl of glucose-6-phosphate 50 mM,

40 μl of Glucose-6-phosphate dehydrogenase 40 U/ml, 80 μl of nitrate reductase from Aspergillus niger 10 U/ml and PBS to 4 ml) were added to each well. All the products for the master mix were purchased from SIGMA. Furthermore, two standard curves were made with known amounts of nitrite and nitrate to determine the efficiency of the conversion from nitrate to nitrite and to quantify the concentrations of the samples. The reaction was developed for 45 minutes at room temperature and then the Griess reaction was performed by adding 100 μl of 1% (w/v) sulfanilamide in 5% phosphoric acid followed by 100 μl of 0,1% (w/v) N-(1-naphtyl)ethylenediamine HCl (NED), after 10 minutes of incubation at room temperature the absorbance was read at 540 nm. NO production was estimated from the concentration of nitrite and nitrate in the incubation medium and the urine samples.

Quantitive RT-PCR assay for iNOS mRNA

iNOS and hypoxanthine-guanine phosphoribosyltransferase (HPRT) transcript mRNA from freshly extracted duodenum, jejunum and ileum was obtained using guanidinium acid-thiocyanate and phenol-chloroform extraction (31). 2 μg of RNA were denatured and reverse transcribed using random hexanucleotides (Pharmacia, Uppsala, Sweden). The real time PCR was performed in duplicate in a volume of 25 μl reactions containing Platinum® SYBR® Green qPCR Supermix-UDG (Invitrogen), forward and reverse primers, and 0.5 μl of cDNA on an ABI Prism® 7500 sequence detection system (Applied Biosystems). The following primer sequences and concentrations (determined following guidelines from Applied Biosystems) were used:

Sense iNOS: 5´ GGC AGC CTG TGA GAC CTT TG 3´ 500 nM

Antisense iNOS: 5´ GCA TTG GAA GTG AAG CGT TTC 3´ 500 nM

Sense HPRT 5´ GCT TTT CGC CGC TTG CT 3´ 500 nM

Antisense HPRT 5´ CTC GTC ATC GGC CGT GAT 3´ 500 nM

Serial-fold dilutions of a cDNA sample were amplified to control amplification efficiency for each primer pair. Thereafter the Ct values for all cDNA samples were obtained. HPRT was used as a control gene to calculate the ΔC_t values for individual samples. The relative amount of iNOS/ HPRT transcripts was calculated using the $2^{(\Delta\Delta Ct)}$ method as described previously (16). These values were then used to calculate the mean of the relative expression (fold induction) of iNOS mRNA in tissues from uninfected and infected mice.

Statistical analysis

In those experiments in which statistical analysis was required the non-parametric Mann-Whitney U test was used to determine the presence or absence of differences between different groups and significant level of $P < 0,05$ was used. All the analyses were performed by using the SPSS package for Macintosh computers (LEAD Technologies Inc., USA).

RESULTS

NSP4 induces a rapid release of NO metabolites from human intestinal cells.

To investigate if human intestinal cells respond with release of NO following incubation with NSP4, confluent monolayers of HT-29 were incubated with various concentrations of NSP4. As shown in figure 1A, the NO_2/NO_3 concentration in the supernatants was increased within 5 minutes after administration of 100 nM of NSP4. Figure 1B shows that the effect of NSP4 was dose-dependent and reached a plateau >100nM. On the contrary, rotavirus infection of HT-29 cells did not result in a detectable NO release, independently of virus concentration or time (data not shown).

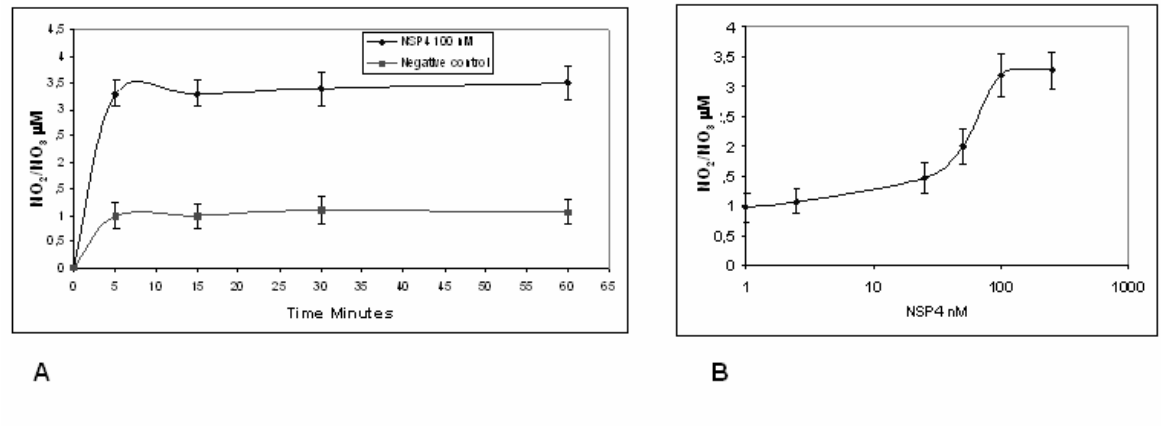

Figure 1. NO_2/NO_3 concentration in the supernatants of HT-29 cells after incubation with the NSP4 toxin. Panel A shows the time-dependent response after incubation with the NSP4 enterotoxin at a concentration of 100 nM. As it can be observed the increase of NO_2/NO_3 in the supernatants occurs within the first five minutes after the addition of the toxin. Panel B shows the dosis-dependent response curve of NO_2/NO_3 after the addition of different concentrations of NSP4. In this experiments the incubation time was 5 minutes in all the cases in base of the previous results. For the negative control purified antigen from Sf9 mock infected cells was used.

Rotavirus stimulates release of NO in vivo

To what extent rotavirus infected release NO was studied in two types of experiments. In a first series of experiments, 7 days old pups were infected with EDIM and diarrhoea and NO_2/NO_3 levels in the urine were recorded. All infected pups responded with diarrhoea following inoculation with EDIM. The results obtained with the newborn mice show two different peaks of NO metabolites release, the first less pronounced approximately at the onset of diarrhoea (figure 2A) and the second more pronounced on day 9 post-infection. Mock infected mice show a steady increase of NO derivatives release during the first 4 days, most likely due to ageing. Watery diarrhoea was starting at day 2 after infection and was resolved at day 11 after infection (figure 2A). The observation that NO metabolites not only were rapidly released at the onset of diarrhoea, but also increased between days 6 and 9, suggest participation of iNOS. Therefore, iNOS mRNA was measured 5 days after infection in the duodenum, jejunum and ileum. While a 3 fold-increase of iNOS mRNA levels in the ileum of the infected

mice was noted no iNOS mRNA increase could be detected in jejunum or duodenum (figure 3).

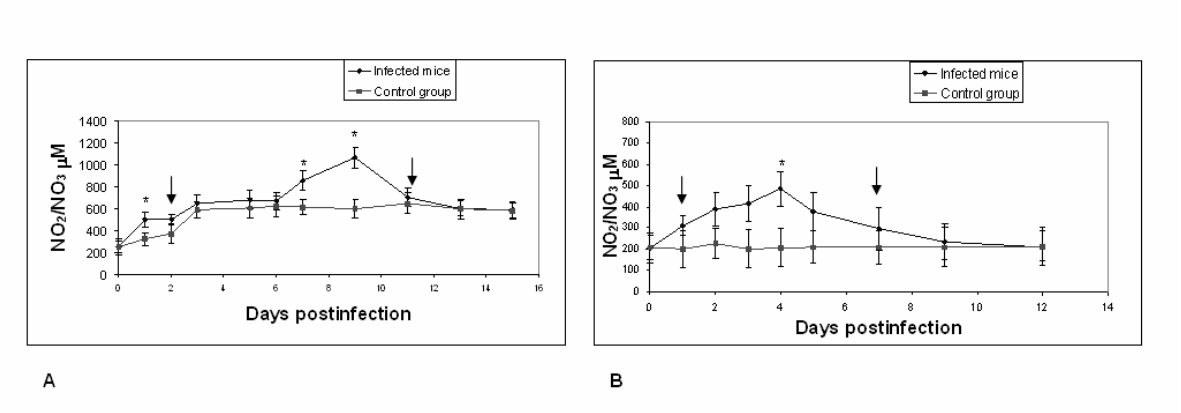

Figure 2. NO_2/NO_3 concentration measured in the urine of the infected mice. Panel A shows the NO_2/NO_3 concentration in the urine of newborn mice (n=10) after rotavirus infection. The arrows show the beginning and the end of the diarrhoea induced by rotavirus and the (*) show the sampling points where statistical differences were observed (*P<0,05*). Two different maximum points can be observed. The first one occurred at 24 h post infection (p.i.) just before the starting of the symptoms at 24 h p.i. A second maximum point occurred at day 9 p.i. very close to the ending of the diarrhoea at day 11 p.i. Panel B shows the NO_2/NO_3 concentration measured in the urine of the adult infected mice (n=10). The arrows show the first and the last day where rotavirus could be detected in the faces and the (*) shows the presence of significant differences (*P<0,05*). A single maximum point occurred at day 4 p.i. and the virus shedding was finally resolved at day 7 p.i.

To study if NO correlated exclusively with clinical symptoms and thus only occurred in newborn pups, adult mice were infected and NO_2/NO_3 and viral shedding recorded in a second type of experiments. Figure 2B shows not only that adult mice respond with release of NO metabolites following infection but also that the concentration in urine increased from day 1 to day 4. The levels of NO_2/NO_3 recovered their basal levels at day 9 after infection. Notably, the kinetics of NO_2/NO_3 levels in urine and of viral shedding (arrows in figure 2B) are parallel (figure 2B).

Table 1. Correlation between NO_2/NO_3 concentration and clinical symptoms from rotavirus disease. [a]Number of children in each group.[b]Total number of children.

Fever status		**Dehydration (DH) status**			**Vomiting status**		**Sex**	
Without fever	With fever	Mild DH	Moderate DH	Severe DH	Without vomits	With vomits	Male	Female
1606,44 µM SD 1114,20 n[a]= 19	1385,76 µM SD 1007,95 n=26	1790,84 µM SD 925,93 n=5	1004,16 µM SD 848,95 n=12	1582,64 µM SD 1115,11 n=29	1189,26 µM SD1000,43 n=22	1756,02 µM SD 1039,45 n=23	1645,84 µM SD 1032,79 n=29	1035,64 µM SD 978,85 n=15
N[b]=45		N=46			N=45		N=44	

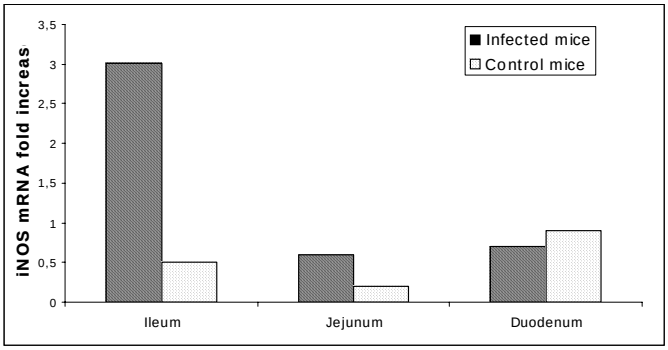

Figure 3. The figure shows the activation of the iNOS mRNA synthesis in the intestine of the newborn mice measured as the fold increase of iNOS mRNA concentration compared to HPRT mRNA in the same sample. A three times fold increase of iNOS mRNA can be observed in ileum, but not in jejenum and duodenum at 5 days post infection.

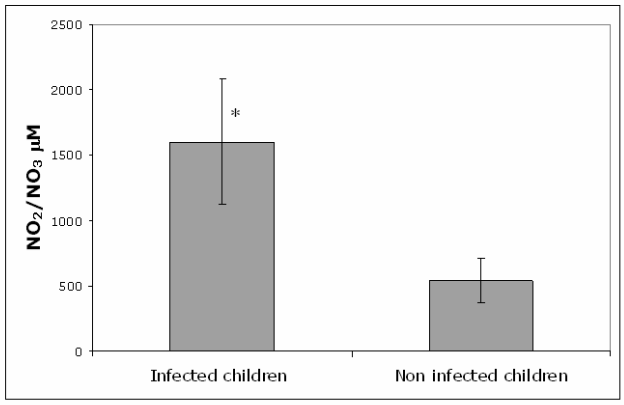

Figure 4. NO_2/NO_3 concentration measured in the urine of rotavirus infected (n=46) and non-infected children (n=19). The (*) shows the presence of significant differences (*p<0,001*) between both groups The bars indicate the standard deviation

Children with severe rotavirus gastroenteritis excrete NO in their urine

In vitro and animal studies suggested an important role of NO during rotavirus infection and disease. To strengthen these observations, a prospective clinical study was performed in Leon, Nicaragua to investigate if also children hospitalized for severe rotavirus disease would release NO. Urine and stool samples from 46 children, less than 2 years of age and 19 healthy age- matched controls were investigated. Figure 4 shows that children with clinical symptoms of rotavirus released significantly more NO derivatives than healthy uninfected children (P<0.001). Efforts were made to correlate the concentrations of NO_2/NO_3 with sex, clinical symptoms or age (table 1). Table 1

shows that the NO_2/NO_3 concentration in the urine within the different groups of children was not related to sex, fever, dehydration status or vomiting.

DISCUSSION

In the present study, we have investigated the role of NO during clinical and experimental infections with rotavirus.

The release of NO metabolites in HT-29 cells after NSP4 exposure exhibited a time-kinetic similar as that of intracellular calcium reported by Dong et al (7). The increase of intracellular calcium in HT-29 cells occurs within seconds after stimulation with NSP4 (7) and is followed by release of NO metabolites as reported in this study. These observations together with the fact that iNOS needs several hours to produce NO after its activation (15) suggest an activation of calcium-dependent cNOS (12, 21). While this had been postulated previously, this is the first time it has been demonstrated that NSP4 can induce secretion of NO in human intestinal epithelial cells.

The effect of NO production by NSP4 was in sharp contrast to the lack of effects after infection of the HT-29 cells with rotavirus. It has been reported that rotavirus infection increases the intracellular calcium concentration (22) and that NSP4 may be implicated in this mechanism (40, 41). However it has also been shown that the intracellular calcium pathway of NSP4 is different depending on administration route. Extracellularly administered NSP4 activate the phospholipase C signalling pathway that release calcium from the ER, while this pathway does not seem to operate when NSP4 is expressed intracellularly (7, 40, 41). The different signalling pathways can possibly explain why extracellulary added NSP4 stimulates secretion of NO in HT-29 cells but not infectious rotavirus.

It has previously been reported that purified NSP4 perturbed polarized epithelia through effects on tight junction (38) and that integrity of tight junction is both calcium

and NO-dependent (44). It is thus reasonable to assume that the effect of NSP4 on polarized epithelial cells include both calcium and NO responses.

As indicated in the introduction an activation of enteric nervous secretory reflex(es) may explain part of the fluid secretion seen in rotavirus infection. It has been proposed that NO released from the intestinal epithelial cells, directly or indirectly, may stimulate enteric nerves (19). The effects of NO on enteric nerve cells have been investigated on myenteric neurons (39). These experiments showed that NO, if anything, hyperpolarizes myenteric neurons, observations that are not compatible with NO directly activating the afferent limbs of secretory reflexes. It seems possible, however, that NO together with e.g. prostaglandins, may activate neurons, since several studies, e.g. reference (3) indicate that NO in conjunction with prostaglandins E_2 may be responsible for inflammatory hyperalgesia. Indeed a role for prostaglandins in rotavirus infection have previously been reported (36) thus suggesting that prostaglandin and NO may activate neurons and induce an inflammatory immune response.

The calcium-independent iNOS is absent in resting cells, but can be induced in macrophages and epithelial cells (1) during infection. Evidence for such mechanisms was also obtained in the present study by showing that intestinal iNOS mRNA was increased, in particular in the ileum, indicating a restricted and not a general activation of iNOS. The results obtained with the murine model showed that rotavirus infection stimulated excretion of NO metabolites in the urine of both adult and newborn mice. The results from the newborn mice suggest that NO may be involved in the pathophysiology of rotavirus, due to the rapid NO production observed at the onset of the diarrhoea. While the observation from adult mice seems to indicate that there is also a participation of NO in the innate immunity as previously reported for other viruses (5, 13, 20, 29). However, further work needs to be done to elucidate whether the activation of NO in the small intestine is involved in the innate immunity against rotavirus or has a direct implication for the pathophysiology of rotavirus induced

secretion by stimulating nervous afferents in the ENS or both. It should be underlined, however, that NO alone was not able to stimulate clinical symptoms, as adult mice did not respond with diarrhoea although the urinary NO_2/NO_3 concentration was increased.

The results from the prospective study strongly suggests a clinical relevance of NO during rotavirus infection. The patients enrolled in this study had all severe gastroenteritis and were hospitalized upon presentation. The concentration of NO_2/NO_3 in the urine (site of accumulation) was high compared to controls and reached similar levels described as for other intestinal diseases (35). Attempts were made to associate sex, age, and clinical symptoms including dehydration with NO_2/NO_3 levels, but no such correlation could be found. A previous report found that children suffering of neuronal diseases linked to rotavirus infections had high levels of NO derivatives in serum and cerebrospinal fluid (11) further supporting a role of NO in rotavirus disease.

In conclusion, the results from in vitro, animal and human studies suggest that NO is an important mediator during rotavirus disease and most likely participate both in the virus pathophysiology and in the immune response.

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DISCUSIÓN

Los objetivos de la presente Tesis Doctoral se han centrado principalmente en el estudio de los aspectos inmunológicos y fisiopatológicos de la glicoproteína NSP4 de rotavirus. Esta proteína ha despertado un gran interés desde que en 1996 fue descrita su actividad enterotoxigénica en el modelo de ratón lactante (Ball et al., 1996). Debido a la trascendencia epidemiológica de las infecciones por rotavirus, tanto en los países de baja renta como en los países desarrollados, es importante conocer cuáles son los determinantes inmunológicos capaces de inducir protección, así como determinar las principales causas fisiopatológicas de la diarrea por rotavirus.

El primero de los objetivos de este estudio fue la producción y purificación de la glicoproteína NSP4 procedente de cuatro cepas diferentes de rotavirus, aisladas tanto de humanos como de animales. En el capítulo 1 ("*Expression and purification of polyhistidine-tagged rotavirus NSP4 proteins in insect cells*") se describe la clonación, producción y purificación de las proteínas NSP4 procedentes de la cepas de rotavirus humanos ITO y Wa (genotipo B), de la cepa porcina OSU (genotipo B) y de la cepa de simio SA11 (genotipo A) en el sistema de expresión de baculovirus. Este sistema fue elegido, en lugar de sistemas más habituales como el de *E. coli*, debido a que la proteína NSP4 posee dos sitios de glicosilación en su extremo amino-terminal. Mediante el sistema de expresión de baculovirus en células de insecto fue posible la producción de las cuatro proteínas en su forma glicosilada. El grado de glicosilación de las proteínas recombinantes no fue en ninguno de los casos del 100%, pero se consiguieron niveles de glicosilación que variaron entre el 40 y el 80% según la proteína producida. Las diferentes proteínas fueron sintetizadas con una cola de 6 histidinas para facilitar su purificación mediante cromatografía de afinidad en columnas de niquel. Con anterioridad ya se había procedido a la clonación, producción y purificación de la proteína NSP4 de la cepa SA11 en baculovirus (Ball et al., 1996), pero el proceso de purificación requería de múltiples pasos de cromatografía. Las proteínas producidas en células de insecto y purificadas mediante cromatografía de

afinidad mostraron conservar sus funciones biológicas, ya que en experimentos de inoculación “in vivo” todas ellas fueron capaces de inducir diarrea en ratones lactantes de 6 días de edad. No obstante, una proteína celular de 90 KD que copurificaba con la proteína NSP4 no fue capaz de inducir diarrea cuando se inoculaba a los ratones recién nacidos. Los resultados obtenidos en este primer trabajo nos permitieron disponer de los reactivos necesarios para continuar nuestro estudio sobre la inmunogenicidad y distintos aspectos fisiopatológicos de la proteína NSP4 de rotavirus.

El segundo objetivo de la presente Tesis fue analizar la estructura antigénica de la proteína NSP4 y abordar la producción de anticuerpos monoclonales que nos permitieran neutralizar la actividad toxigénica de la proteína NSP4. La proteína NSP4 purificada fue utilizada para inmunizar ratones Balb/c de 4 semanas de edad. La inmunización se realizó utilizando protocolos estándar y se obtuvieron varios sueros hiperinmunes frente a la proteína NSP4 de las diferentes cepas utilizadas. Pese a que los títulos de los sueros de los diferentes ratones inmunizados fueron elevados en todos los animales inmunizados con distintas proteínas, y a que se obtuvieron varios hibridomas productores de anticuerpos monoclonales frente a NSP4, durante el proceso de purificación por dilución límite se comprobó la inestabilidad de todos ellos. Existe una nueva metodología que permite la producción de anticuerpos monoclonales denominada la técnica de “*phage display*”. En el capítulo 2 de la presente Tesis (“*Isolation and characterization of single-chain variable fragment (scFv) antibodies against rotavirus NSP4 and VP8* proteins generated by phage display*”) se describe la producción de anticuerpos de simple cadena frente a las proteínas NSP4 y VP8* de rotavirus mediante la técnica de “*phage display*”, consiguiendo de este modo la producción y caracterización de anticuerpos monoclonales a pesar de los resultados negativos obtenidos utilizando la técnica de producción de hibridomas. Los anticuerpos monoclonales de simple cadena (scFv, del inglés “*single chain variable fragments”*)

son proteínas quiméricas que se producen tras fusionar las zonas variables da las cadenas pesadas (V_H) y ligeras (V_L) de las inmunoglobulinas y poseen capacidad de reconocimiento de epítopos idéntico al de las inmunoglobulinas naturales, aunque carecen del fragmento cristalizable y de las propiedades que confiere el hecho de poseer una estructura multimérica. A pesar de las carencias que estos anticuerpos presentan con respecto a las inmunoglobulinas naturales, estos reactivos monoclonales han demostrado ser útiles a la hora de bloquear la infectividad de otros virus (de Carvalho Nicacio et al., 2002).

En el primero de los trabajos presentados en el capítulo 2 (capítulo 2A "*Single-chain variable fragment (scFv) antibodies against rotavirus NSP4 enterotoxin generated by phage display*") la técnica de "*phage display*" fue utilizada con éxito y se obtuvieron diferentes anticuerpos de simple cadena frente a la proteína $NSP4_{wa}$. Tras la caracterización molecular de los diferentes clones, se identificaron un total de 4 anticuerpos en base al análisis de restricción y a las diferencias en la secuencia de los diferentes clones. Se analizó su capacidad de reconocer epítopos lineales en la proteína NSP4 mediante western blot. Tres de los clones (2B5, 2C3 y 2D1) fueron capaces de reaccionar en western blot, lo que indica que reaccionan frente a epítopos lineales, mientras que el clon 2B12 fue negativo, sugiriendo que el epítopo reconocido por este clon es aparentemente conformacional. El siguiente paso en el análisis de los diferentes clones fue determinar qué región de la proteína NSP4 reconocía cada uno de ellos. Con este propósito se produjeron en *E. coli* cuatro construcciones de la proteína $NSP4_{Wa}$ como proteínas de fusión a GST para permitir su purificación por cromatografía de afinidad. Las construcciones consistían en tres formas truncadas de la proteína $NSP4_{Wa}$ en su extremo carboxi-terminal y una cuarta con la proteína completa. Tres de los clones obtenidos (2B5, 2C3 y 2D1) mostraron ser capaces de reconocer la región de la proteína que se encuentra delimitada entre los aminoácidos 91 y 135, que contienen la fracción toxigénica de la proteína (aminoácidos 114-135). El clon 2B12 sólo fue capaz de reconocer la proteína completa expresada en *E. coli,*

indicando que los aminoácidos 135 a 175 de la proteína se encuentran implicados en la formación del posible epítopo conformacional reconocido por este anticuerpo. Este resultado contrasta con los resultados obtenidos con el suero policlonal de ratón producido frente a la misma proteína en nuestro laboratorio, ya que este suero posee una reactividad muy pobre frente a la región delimitada entre los aminoácidos 91 y 135 de la proteína, siendo la región carboxi-terminal de la proteína (aminoácidos 135-175) la más inmunogénica. Los resultados obtenidos con nuestro suero policlonal están en concordancia con los obtenidos por Borgan y colaboradores con la proteína NSP4 de la cepa aviar de rotavirus PO13, en la que tras estudios de inmunización demostraron que la región carboxi-terminal de la proteína que se encuentra a continuación del péptido toxigénico es inmunodominante (Borgan et al., 2003). Los resultados obtenidos con la técnica de "*phage display*", en la que el 75% de los clones obtenidos reaccionan frente a una región no inmunodominante de la proteína, pueden deberse a que en el proceso de selección de los scFv no existe ningún sistema inmunológico implicado, de forma que los clones que no podrían ser obtenidos "in vivo" son obtenidos mediante esta técnica. Finalmente, y para completar la caracterización de los scFv obtenidos, se estudió su capacidad para reconocer proteínas NSP4 de diferentes genotipos. Se utilizó la técnica de ELISA para determinar la reactividad de los 4 clones obtenidos frente a las proteínas NSP4 procedentes de la cepa Wa (genotipo B y proteína con la cual se obtuvieron los scFv) y de la cepa SA11 (genotipo A). Tres de los clones (2B5, 2B12 y 2C3) reaccionaron frente a ambas proteínas, indicando el reconocimiento de epítopos conservados en ambas proteínas de genotipos A y B. No obstante, el clon 2D1 sólo fue capaz de reconocer la proteína $NSP4_{Wa}$, indicando que reconoce un epítopo no conservado en ambos genotipos. Este último resultado plantea la posibilidad de establecer una clasificación antigénica de las proteínas NSP4, aunque sería necesaria una caracterización más detallada con proteínas de los diferentes genotipos de NSP4 descritos hasta el momento.

Uno de los principales objetivos planteados al comienzo de la presente Tesis fue analizar la presencia de epítopos neutralizantes en la proteína NSP4. Pese al esfuerzo realizado en esta dirección y debido a la falta de un buen modelo “in vitro” y a la dificultad que implica el modelo “in vivo” para probar la capacidad neutralizante de los reactivos monoclonales obtenidos, no fue posible determinar la capacidad neutralizante de los mismos. No obstante, y prácticamente en paralelo, obtuvimos una colección de scFv frente a la fracción VP8* de la proteína VP4 de rotavirus en colaboración con el Departamento de Biotecnología del Instituto de Agroquímica y Tecnología de los Alimentos (IATA, CSIC). El segundo trabajo presentado en el capítulo 2 (capítulo 2B “*Selection of single-chain antibodies against the VP8* subunit of rotavirus VP4 outer capsid protein and their expression in Lactobacillus casei*”) muestra la selección, caracterización y expresión en *L. casei* de scFv también obtenidos mediante la técnica de “*phage display*” y procedentes de la misma genoteca utilizada en el capítulo 2A. La principal razón por la que se presenta este trabajo en esta Tesis, pese a que no se centra en el estudio de la proteína NSP4, es porque en él se demuestra que los anticuerpos de simple cadena producidos mediante la técnica de “*phage display*” pueden poseer capacidad neutralizante, como indican los resultados obtenidos en el capítulo 2B. Un total de 8 scFv diferentes frente a la proteína VP8* de rotavirus fueron producidos y caracterizados de forma similar a los scFv obtenidos frente a la proteína NSP4. Todos ellos fueron analizados para conocer su capacidad para neutralizar la infectividad por rotavirus. Con ese propósito se utilizó un ensayo de neutralización “in vitro”. Los resultados indican que todos los scFv obtenidos fueron capaces de neutralizar la infección por rotavirus en células MA104, no así un fago M13 que expresaba un scFv sin capacidad de reconocimiento para la proteína VP8*. El nivel de neutralización fue diferente en cada uno de los clones obtenidos, pero los clones 2A1 y 2A12 presentaron porcentajes de inhibición superiores al 70%. Por este motivo el clon 2A1 (porcentaje de inhibición superior al 80%) fue elegido para su expresión en la bacteria láctica *Lactobacillus casei.* La expresión de la proteína en *L.*

casei tuvo como principal inconveniente que la concentración de scFv producido en ella no era suficiente para realizar estudios de inhibición “in vivo”. No obstante, los resultados obtenidos muestran que el scFv producido por *L. casei* presenta la conformación adecuada, ya que mantiene su capacidad de reconocimiento frente a la proteína VP8* de rotavirus. A pesar del pobre nivel de expresión, los resultados obtenidos en este trabajo abren la posibilidad de utilizar bacterias consideradas seguras como vectores para inducir inmunidad pasiva y bloquear la infectividad de rotavirus “in vivo.”

El tercero de los objetivos de la presente Tesis fue determinar la importancia de la respuesta inmune frente a la proteína NSP4 en humanos para analizar la posible relación entre la presencia o ausencia de anticuerpos frente a esta proteína vírica y la protección frente a la enfermedad producida por la infección por rotavirus. En el capítulo 3 (“*Humoral immune response to the NSP4 enterotoxin of rotavirus in Spanish children*”) se aborda el estudio en el que se determina la presencia y el título de anticuerpos frente a las proteínas $NSP4_{Wa}$ y $NSP4_{SA11}$ (genotipos B y A respectivamente) presentes en el suero de niños convalecientes de una infección por rotavirus, niños sanos (supuestamente susceptibles a padecer diarrea por rotavirus) y adultos sanos (en principio protegidos frente a la diarrea por rotavirus). Los resultados obtenidos en el trabajo muestran que existe una alta prevalencia (70%) de anticuerpos de clase IgG, pero no de clase IgA, frente a NSP4 en los sueros de los niños convalecientes con títulos bajos o muy bajos. No obstante tanto en los niños sanos como en los adultos sanos el porcentaje de sueros con capacidad de reaccionar frente a la proteína NSP4 fue muy bajo en ambos casos (26 y 18% respectivamente) y siempre a títulos bajos o muy bajos. En estos dos grupos tampoco fueron detectados anticuerpos séricos de clase IgA específicos de NSP4. Estos resultados contrastan con los resultados obtenidos frente a la partícula completa de rotavirus, en los que en todos los grupos estudiados la prevalencia de anticuerpos fue superior al 80%

indicando seroconversión en los niños convalecientes y contacto previo con el virus en los niños y adultos sanos. Otro de los aspectos importantes sobre la inmunidad conferida por la proteína NSP4 en infecciones naturales es conocer si existe reacción cruzada entre los diferentes genotipos de la proteína NSP4. En este estudio la proteína NSP4 de los dos genotipos más comunes en humanos (A y B) (Iturriza-Gomara et al., 2003) fueron estudiados para determinar la reactividad cruzada entre ambos genotipos. Los resultados indican que el 40% de los sueros de los niños convalecientes fueron capaces de reconocer la proteína NSP4 de ambos genotipos A y B. Pese al alto porcentaje de reactividad cruzada observada, el análisis estadístico muestra que sí existen diferencias significativas en el reconocimiento de ambas proteínas por los diferentes sueros analizados. Los resultados obtenidos en este estudio pueden ser comparados con los obtenidos en estudios similares en diferentes países. Nuestros resultados concuerdan en casi todos los aspectos con los obtenidos por Johansen y cols. en 1999, con la única diferencia de que ellos detectan una alta prevalencia de anticuerpos frente a NSP4 en la población adulta. No obstante un trabajo más reciente realizado en niños hospitalizados en la India (Ray et al., 2003) presenta resultados muy diferentes de los nuestros. En el estudio presentado por Ray y cols. los títulos de anticuerpos de clase IgG son mucho más elevados que los hallados por nosotros y por Johansen y cols.; así mismo en dicho trabajo se muestran títulos de anticuerpos de clase IgA similares a los de anticuerpos clase IgG, mientras que nosotros no encontramos anticuerpos IgA, al igual que Johansen y colaboradores. La siguiente diferencia entre nuestro trabajo y el trabajo presentado por Ray y colaboradores es que ellos demuestran que existe reconocimiento cruzado entre los diferentes genotipos de la proteína NSP4.

En el cuarto capítulo, "*Nitric oxide production during clinical and experimental infection with rotavirus*" se describe un nuevo aspecto de la actividad fisiopatológica de la proteína NSP4. En este capítulo se realiza un estudio que comienza con los

experimentos “in vitro” realizados sobre células epiteliales humanas HT-29 y termina con experimentos realizados con muestras clínicas. Hasta el momento, varios trabajos habían propuesto la participación del óxido nítrico (ON) en la patogénesis de rotavirus (Lundgren y Svensson, 2001; Morris y Estes, 2001), hipotetizando que las células epiteliales infectadas por rotavirus podrían sintetizar dicha molécula que actuaría estimulando el sistema nervioso entérico (SNE). Los experimentos realizados “in vitro” muestran que la proteína NSP4 posee la capacidad de estimular la secreción de productos derivados del ON al medio de cultivo. Esta secreción ocurre durante los primeros minutos tras la adición de NSP4 y se trata de una respuesta dependiente de la concentración. Estos resultados coinciden con el incremento de calcio intracelular que se observa en las mismas células HT-29 en respuesta a la toxina NSP4 (Dong et al., 1997) indicando que la enzima implicada es la óxido nítrico sintasa constitutiva (cNOS) dependiente de calcio. Se realizaron experimentos infectando con rotavirus las células HT-29, pero paradójicamente en las células infectadas no se observó una respuesta de ON. De esta forma, el incremento de calcio intracelular inducido por la toxina NSP4 cuando actúa extracelularmente produciría, además de los demás efectos descritos hasta el momento, una activación de la enzima cNOS, produciéndose así un aumento en los niveles de ON secretados por las células. En un segundo grupo de experimentos se estudió la secreción de productos derivados del ON “in vivo” en el modelo murino. Los resultados obtenidos indican que “in vivo” rotavirus es capaz de incrementar la concentración de los derivados del ON en la orina de los ratones infectados. Este incremento se observa tanto en el modelo de ratón lactante como en los ratones adultos, y en ambos casos existe una relación directa con la diarrea y con la excreción de rotavirus, respectivamente. Este incremento de ON “in vivo” está relacionado con la activación de la enzima óxido nítrico sintasa inducible (iNOS), como demuestran los resultados por PCR a tiempo real para detectar los niveles de ARN mensajero de la enzima iNOS en el intestino de ratones lactantes infectados. Finalmente, se utilizaron muestras de orina provenientes de niños con infección por

rotavirus y de niños sanos. Los resultados obtenidos con dichas muestras indican que existe un incremento en la secreción de los derivados del ON en la orina de los niños infectados por rotavirus. Estos resultados confirman la implicación del ON en la fisiopatología de la infección por rotavirus, no sólo en el modelo murino sino también en humanos.

CONCLUSIONES

Conclusiones

1. El sistema de expresión de baculovirus en células de insecto es apropiado para la producción y purificación de la proteína NSP4 de diferentes cepas de rotavirus humanas y de animales en forma biológicamente activa.

2. Los sueros obtenidos de ratones inmunizados con la proteína NSP4 reconocen principalmente el fragmento carboxi-terminal de la proteína (aminoácidos 114 a 175), por lo que esta región es inmunodominante.

3. Los anticuerpos producidos mediante la técnica de "*phage display*" son reactivos útiles para identificar epítopos en la proteína NSP4 y probablemente para realizar estudios de inhibición de sus funciones biológicas.

4. La utilización de la técnica de "*phage display*" permite el aislamiento de anticuerpos monoclonales de simple cadena frente a regiones no inmunodominantes de la proteína NSP4.

5. La proteína NSP4 provoca una respuesta inmune humoral tras la infección natural por rotavirus en niños, constituída por anticuerpos séricos de clase IgG. Esta respuesta no parece ser de larga duración, según los resultados obtenidos con sueros de niños y adultos sanos.

6. Los anticuerpos IgG séricos desarrollados frente a NSP4 en niños con gastroenteritis aguda por rotavirus presentan en el 40% de los casos estudiados reactividad cruzada frente a más de un genotipo de dicha proteína, por lo que este reconocimento de los genotipos A y B de NSP4 no es siempre heterotípico.

7. La proteína NSP4 provoca un incremento de la secreción de óxido nítrico en cultivos de células epiteliales humanas HT-29. Este incremento ocurre de forma paralela al incremento de calcio intracelular observado en la misma línea celular en respuesta a NSP4, indicando una posible participación de la enzima óxido nítrico sintasa constitutiva.

8. Las infecciones por rotavirus en ratones Balb/c provocan un aumento en la concentración de los productos derivados del óxido nítrico en la orina de los animales infectados, con la participación de la enzima óxido nítrico sintasa inducible en el modelo murino. Este incremento se observa también en la orina de niños con gastroenteritis por rotavirus.

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