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Caracterización de la respuesta inmune de lechones durante la infección y tras la vacunación con el virus del Síndrome Reproductivo y Respiratorio Porcino

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HAGO CONSTAR,

Que el trabajo "Caracterización de la respuesta inmune de lechones durante la infección y tras la vacunación con el virus del Síndrome Reproductivo y Respiratorio Porcino", presentado por Ivan Díaz Luque para la obtención del grado de Doctor en Medicina y Sanidad Animal, ha sido realizado en dicho departamento y bajo mi dirección.

Para que conste, firmo la presente,

Enric Mª Mateu de Antonio

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Introducción

Hay que estudiar mucho para saber poco

Charles de Montesquieu

1. INTRODUCCIÓN

1.1 BREVE HISTORIA DEL SÍNDROME REPRODUCTIVO Y RESPIRATORIO PORCINO

El Síndrome Reproductivo y Respiratorio Porcino (PRRS)[1] se describió por primera vez en los Estados Unidos de América a finales de la década de 1980 (Keffaber, 1989; Loula, 1991) y en Europa pocos años después (Lindhaus y Lindhaus, 1991).

El primer brote conocido apareció en granjas del estado de Carolina del Norte en 1987 y se caracterizó por oleadas de abortos, un aumento de los nacidos muertos y mortalidad neonatal. Posteriormente, también se observaron graves problemas respiratorios en cerdos destetados de cualquier edad (Hill, 1990). En 1990, cerca de Münster (Alemania), se detectaron brotes de una enfermedad que mostraba signos clínicos parecidos (Lindhaus y Lindhaus, 1991). Más tarde aparecieron casos similares en el Reino Unido, Holanda y Francia (White, 1991; Baron *et al*., 1992; Meredith, 1992).

El primer caso en España se detectó en enero de 1991 en la provincia de Huesca. El brote se caracterizó por un cuadro respiratorio agudo que causó la muerte de 74 cerdos de un lote de 300 cerdos de engorde. Los animales afectados procedían de Alemania (Plana-Duran *et al*., 1992a). En marzo del mismo año, dos granjas de ciclo cerrado cercanas al primer brote presentaron problemas reproductivos caracterizados por un elevado número de abortos y una mortalidad del 70% en lechones (Plana-Duran *et al.,* 1992b). Tras estos casos la enfermedad fue diseminándose por todo el país.

Inicialmente la enfermedad fue llamada “Mystery Swine Disease” (Enfermedad misteriosa del cerdo), por el desconocimiento que se tenía del agente causante de la misma (Hill, 1990). Este nombre fue variando en función de la importancia que se otorgaba a uno u otro signo clínico (enfermedad de las orejas azules, síndrome disgenésico y respiratorio del cerdo, etc.) (Benfield *et al.,* 1999; Zimmerman *et al.,* 2003a). Finalmente, en el I Congreso Internacional de esta

[1] En la presente tesis se utilizará el acrónimo en inglés: PRRS de “Porcine Reproductive and Respiratory Syndrome”

enfermedad (17 de Mayo de 1992, St. Paul, Minnesota, EEUU) se aceptó el nombre con el que se conoce en la actualidad.

El agente causal de la enfermedad, el virus del PRRS (PRRSV), fue aislado por primera vez en el año 1991 por el *Central Veterinary Research Institute* en Lelystad (Holanda) usando cultivos de macrófagos alveolares de porcino (Wensvoort *et al*. 1991). Más tarde, el mismo agente se aisló de los brotes presentes en EEUU usando la línea celular CL-2621 (*Boehringer Ingelheim Animal Health Inc*.) (Collins, 1991; Collins *et al*., 1992). Desde un principio se observó que se trataban de dos genotipos diferentes. La cepa aislada en Holanda fue denominada virus de Lelystad (LV) y pasó a convertirse en la cepa de referencia del genotipo europeo, mientras que la aislada en los EEUU recibió el nombre de ATCC-VR-2332 y se consideró como la cepa de referencia del genotipo americano.

El origen del PRRSV es incierto. Actualmente, una de las hipótesis más aceptadas sobre su origen es la planteada por Plagemann (2003). Según este autor, mutaciones en el virus elevador de la lactato-deshidrogenasa del ratón (LDV) habrían dado lugar a un eslabón intermedio entre este virus y el del PRRS. Una especie que tuviera un contacto natural tanto con el ratón como con el cerdo doméstico, probablemente el jabalí, habría actuado de reservorio de dicho eslabón. La exportación, a principios del siglo XX, de jabalíes desde Europa hasta un coto de caza de Carolina del Norte habría llevado el eslabón de un continente a otro. Así, durante aproximadamente 60 años, ambos virus habrían evolucionado de forma separada, lo que explicaría la presencia en la actualidad de dos genotipos diferenciados (americano y europeo). Los cambios en el manejo y la estructuración de la producción porcina en el último cuarto del siglo XX habrían facilitado la emergencia de la enfermedad en ambos continentes (Nelsen *et al*., 1999). Esta hipótesis coincide con los análisis retrospectivos de sueros y con los estudios realizados a partir de cálculos en la tasa de mutación. Según estos estudios, el virus entró y se habría adaptado a la población del porcino doméstico entre finales de la década de 1970 y mediados de 1980, según localización (Shin *et al*., 1993; Carman *et al*., 1995; Hirose *et al*., 1995; Ohlinger *et al*., 2000; Forsberg *et al*., 2001; Mateu *et al*. 2006).

Desde la aparición de los primeros brotes, el impacto económico de la enfermedad en la cabaña mundial porcina ha sido y es muy importante (Polson *et al*., 1992). El último estudio realizado en los EEUU estima unas pérdidas anuales de 560,32 millones de dólares a causa de los costes directos derivados de la presentación aguda de esta enfermedad (Neumann *et al*., 2005).

1.2 AGENTE ETIOLÓGICO

1.2.1 Clasificación taxonómica

El PRRSV, también conocido como Arterivirus porcino, está incluido en el género *Arterivirus,* en la familia *Arteviridae.* En este género también están incluidos el virus de la arteritis equina, el virus de la fiebre hemorrágica del simio y el LDV (Meulenberg *et al.,* 1994). La familia *Arteriviridae*, junto a la familia *Coronoviridae* y *Roniviridae,* pertenece al orden de los Nidovirales (Cavanagh, 1997). Los cuatro miembros del género *Arterivirus* comparten propiedades biológicas y moleculares únicas: organización del genoma, gran variabilidad genética, estrategia de replicación, secuencia de trascripción, composición proteica, morfología del virión, especificidad celular muy restringida (replicación primaria en macrófagos) y capacidad de provocar desde infecciones asintomáticas, asintomáticas persistentes o hasta casos clínicos desde leves a graves e incluso fatales (Snijder y Meulenberg, 1998; Balasuriya y MacLachlan, 2004).

1.2.2 Organización genómica y proteica

El virión completo es de tamaño pequeño, esférico y con envoltura. El tamaño de la partícula viral oscila entre 48 y 83 nanómetros, mientras que la nucleocápside, de simetría icosaédrica, oscila entre los 25 y 30 nanómetros (Benfield *et al*., 1992; Botner *et al*., 1994; Mardassi *et al*., 1994; Dea *et al*., 1995). En su interior, el genoma está formado por una única cadena de RNA de 15.100 bases, de polaridad positiva, no segmentada y poliadenilada (Meulenberg *et al*., 1993; Conzelmann *et al*., 1993). Está cadena está compuesta por nueve fragmentos de lectura abierta (o ORF acrónimo correspondiente a *Open Reading Frames)* que se solapan parcialmente entre sí (Conzelmann *et al*., 1993; Meulenberg *et al*., 1993; Allende *et al*., 1999; Shen *et al*., 2000) (Figura 1). Las ORFs 1a y 1b ocupan 12.000 pares de bases, aproximadamente el 80% del genoma, y codifican para proteínas no estructurales que participan en la transcripción y replicación del RNA, incluyendo la RNA-polimerasa. El resto del genoma lo componen las ORFs que codifican para las proteínas que forman el virión (ORFs 2-7) (Snijder y Meulenberg, 1998, Wu *et al*., 2001).

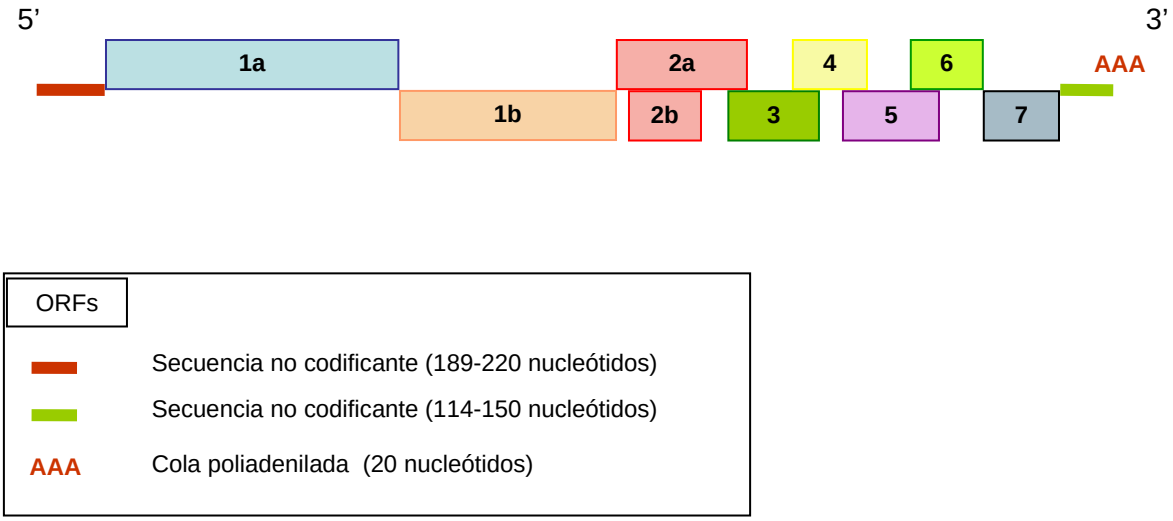

Figura 1. Estructura del genoma del PRRSV. Las ORFs están flanqueadas por dos secuencias no codificantes en ambos extremos del genoma. En el extremo 3' se localiza una cola poliadenilada.

El virión está constituido por tres proteínas estructurales mayores; la glicoproteína 5 (GP5) (codificada por la ORF 5), la proteína M, que constituye la membrana (codificada por la ORF 6), y la proteína N, que constituye la nucleocápside (codificada por la ORF 7); por tres glicoproteínas menores (GP2a, GP3, GP4, codificadas por la ORF 2a, 3 y 4, respectivamente) y por una pequeña proteína no glicosilada llamada 2b (Meulenberg *et al*., 1995a, 1995b; Meulenberg y den Besten, 1996; Van Nieuwstadt *et al*, 1996; Wu *et al.,* 2005) (Figura 2).

En el virión, la GP5 y la proteína M forman un heterodímero con uniones de puentes disulfuro que podría estar involucrado en la unión al receptor celular (Mardassi *et al*., 1996). La proteína N contiene el epítopo inmunodominante (Yoon *et al*., 1995; Plana-Duran *et al*., 1997b) mientras que la GP5 contiene el epítopo neutralizante más importante (Pirzadeh y Dea, 1997; Weiland *et al*., 1999; Plagemann, 2003).

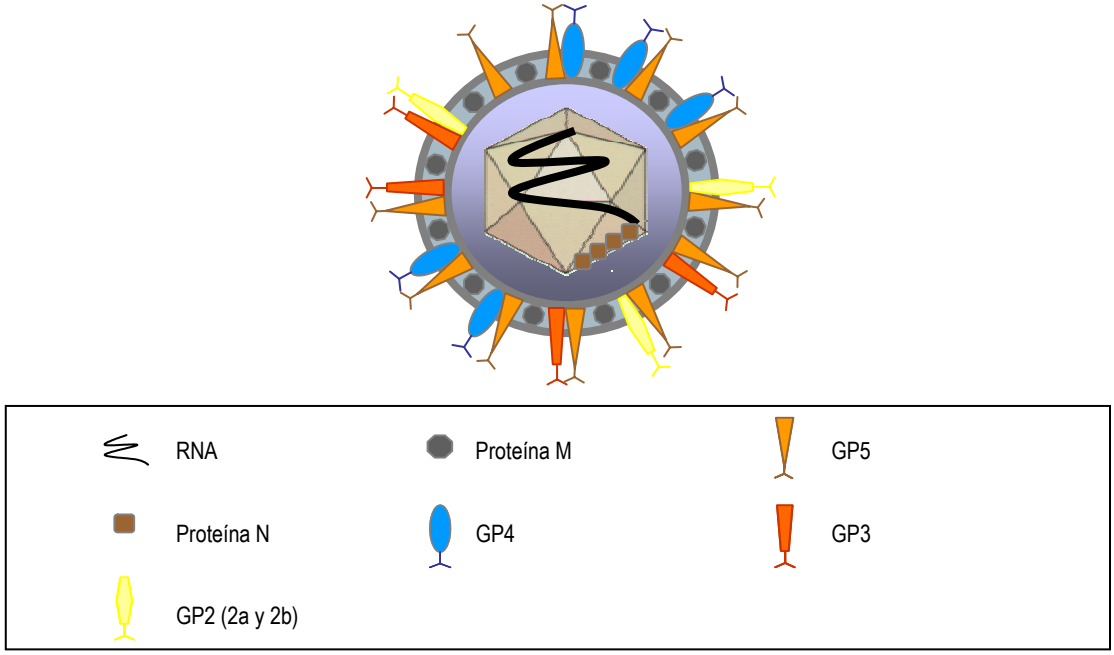

Figura 2. Representación esquemática de la estructura de una partícula de PRRSV y situación de las proteínas estructurales.

1.2.3 Características fisicoquímicas y biológicas

La densidad de flotación del PRRSV en $CsCl_2$ es de 1.13-1.19 g/ml y de 1.18-1.23 g/ml en gradientes de sacarosa (Wensvoort *et al.,* 1991; Benfield *et al*., 1992; Bautista *et al*., 1996).

Es un virus bastante lábil en condiciones desfavorables de temperatura y pH, por lo que no suele sobrevivir largos periodos de tiempo fuera del hospedador. Pierde el 90% de su poder infectivo en una semana cuando se conserva a 4º C; sólo persiste durante 1-6 días a 21º C, de 3-48 horas a 37º C y apenas 20-45 minutos a 56º C (Benfield *et al*., 1992; Benfield *et al.,* 1999). Su infectividad disminuye drásticamente, hasta el 90%, a pH menores de 6 y mayores de 7,5 (Benfield *et al*., 1992; Bloemraad *et al*., 1994). Además, se inactiva rápidamente en superficies secas (Pirtle y Beran, 1996).

Los procedimientos típicos de limpieza y desinfección son suficientes para inactivarlo. Es sensible al tratamiento con cloroformo, éter y a soluciones con bajas concentraciones de detergentes (Benfield *et al*., 1992, Bloemraad *et al*., 1994; Plagemman, 1996).

El PRRSV no posee capacidad hemaglutinante (Benfield *et al*., 1992; Dea *et al*., 1992), pero el tratamiento de algunas cepas con detergentes no iónicos y disolventes lipídicos podría favorecer dicha capacidad (Jusa *et al*., 1996).

El PRRSV posee un rango de células para su replicación muy restringido. Se replica principalmente en las células de la línea monocito/macrófago. Concretamente los macrófagos alveolares porcinos (MAP) y los macrófagos intravasculares del pulmón (MIP) se consideran las células diana (Wensvoort *et al.,* 1991; Voicu *et al.,* 1994; Rossow *et al.,* 1995 y 1996; Thanawongnuwech *et al.,* 2000). También puede replicarse en algunas líneas celulares específicas de mono: MARC-145, CL-2621 o CRL11171 (Benfield *et al*., 1992; Kim et al., 1993; Meng *et al.,* 1994 y 1996). La replicación se inicia con la expresión del gen de la replicasa, puede llevarse a cabo en tan sólo 10-12 horas e implica la formación de seis o siete RNA mensajeros, según cepa (Conzelmann *et al*., 1993; Meng *et al*., 1994, 1996; Meulenberg y den Besten, 1996). El RNA mensajero "extra" correspondería a un segmento entre la ORF 3 y la ORF 4 (Meng *et al*., 1996).

En la replicación únicamente participa la RNA polimerasa, no siendo requerida la síntesis de DNA celular (Benfield *et al*., 1992). Aparte de la RNA polimerasa, codificada por las ORF 1a y 1b, Verheije *et al*. (2002) observaron que un segmento altamente conservado dentro de la ORF 7, situado cerca de la terminación 3', es también esencial para la replicación del virus.

1.2.4 Variabilidad genética

Desde que se aisló el agente causal de la enfermedad, se ha determinado la existencia de dos genotipos, el americano y el europeo. Ambos virus producen signos clínicos similares (Benfield *et al.,* 1999). Sin embargo, existe una importante diferencia genética entre ambos. Dependiendo de la ORF examinada, las homologías pueden variar entre el 50% y el 90% (Meng *et al*, 1994). Las similitudes a nivel aminoacídico se sitúan entorno al 55%-80% (Meng *et al*., 1994; Murtaugh *et al*., 1995; Meulenberg *et al*., 1997).

Los análisis genéticos de las cepas aisladas han permitido determinar que los dos genotipos presentan una elevada diversidad genética debido a su alta tasa de mutación (Dea *et al*., 1996; Halbur *et al*., 1996; Kapur *et al*., 1996; Mengeling *et al*., 1996; Goldberg *et al*., 2000; Meng, 2000; Wootton *et al*., 2000; Mateu *et al*., 2003; Mateu *et al.,* 2006). Esta elevada variabilidad puede

estar asociada a dos fenómenos. En primer lugar las RNA polimerasas del virus no poseen la capacidad de corrección de los errores que se producen en el proceso de duplicación del RNA; se considera que pueden aparecer de 2 a 15 mutaciones aleatorias por cada nuevo virus producido (Domingo y Holland, 1997). En segundo lugar, podría existir cierta capacidad de recombinación entre cepas parecidas (Van Vugt *et al*., 2001; Murtaugh *et al*., 2001). Por otro lado, numerosos estudios han demostrado que en el curso de una infección se producen miles de variantes que coexisten dentro de un mismo animal y que dan lugar a la aparición de "cuasiespecies" (Kapur *et al*., 1996; Rowland *et al*., 1999; Goldberg *et al.,* 2003).

Durante una infección experimental los genes que codifican para las proteínas estructurales presentan tasas de mutación mayores que la ORF 1. Concretamente, las ORFs 3, 4 y 5 mutan más que las ORFs 2, 6 y 7. Se ha sugerido que esto sucede por la presión del sistema inmunitario, ya que las proteínas internas presentan menos cambios (Le Gall *et al*., 1998; Weiland *et al*., 1999; Allende *et al*., 2000; Chang *et al*., 2002; Wissink *et al*., 2003). Sin embargo algunos datos indican que, por ejemplo en la ORF 5, muchas de las variantes detectadas presentan cambios importantes en zonas de transmembrana, por lo tanto en zonas que es poco probable que actúen como epítopos de las células B (Hanada *et al.,* 2005; Mateu *et al.,* 2006).

Se considera que la elevada variabilidad genética de este virus repercute negativamente en las respuestas cruzadas (van Woesel *et al.,* 1998) y que además podría estar relacionada con fenómenos de atenuación y adquisición de virulencia (Allende *et al*., 2000; Nielsen *et al*., 2001). Basándose en estudios teóricos, se ha determinado que la ORF 5, la más variable de las ORFs (Forsberg *et al.,* 2002; Mateu *et al*., 2003; Pesch *et al.,* 2005), podría presentar seis sitios potencialmente antigénicos (Andreyev *et al*., 1997; Pirzadeh *et al*., 1998). Así pues, la variabilidad genética de esta ORF podría determinar la pérdida o alteración de algún epítopo neutralizante o de las zonas de glicosilación y por tanto provocar el enmascaramiento de epítopos neutralizantes (Weiland *et al*., 1999; Chang *et al*., 2002; Wissink *et al*., 2003; Plagemann, 2004). Rowland *et al.,* (1999) describieron que tras la infección con la cepa americana ATCC-VR-2332 aparecían nuevas variantes del virus que presentaban cambios en la GP5. Estos cambios provocaron alteraciones en el reconocimiento por parte de la respuesta inmune de los epítopos de neutralización del virus.

1.3 PATOGENIA Y VIRULENCIA

Una de las vías de entrada más importantes del PRRSV en el cerdo es la oro-nasal. Tras la infección de los macrófagos alveolares, lugar primario de replicación del virus, éste se localiza en los tejidos linfoides próximos y finalmente se disemina por todo el organismo (Rossow et al., 1995; Rossow et al., 1996). El estado de viremia se inicia 12 horas post-infección, se considera bastante prolongado y con una marcada variabilidad individual, detectándose hasta 2-4 semanas post-infección en animales adultos y hasta 3 meses en los lechones (Benfield *et al.,* 1997; Van der Linden *et al.,* 2003; Zimmerman *et al.,* 2003b). Tras la viremia puede existir persistencia de la infección.

Se considera que la infección de las cerdas cerca de la concepción puede provocar la infección transplacentaria de los lechones. En el caso de una infección *in utero*, los lechones pueden morir durante la gestación, dando lugar a nacidos muertos o a fetos momificados, nacer débiles o incluso no presentar ningún tipo de sintomatología, lo que da lugar al nacimiento de animales aparentemente sanos pero persistentemente infectados (Albina *et al*., 1994; Wills *et al*., 1995). La infección transplancentaria de los lechones tendría lugar sólo después de su implantación en el útero (Prieto *et al.,* 1997). En consecuencia, según Lager *et al.* (1996) y Prieto *et al.* (1997), la infección de las cerdas gestantes no interfiere de forma notoria con la fertilidad. Contrariamente, otros autores han relacionado la infección de las cerdas gestantes con la reducción de los porcentajes de concepción (Keffaber, 1989; Hopper *et al.,* 1992). Esta aparente contradicción podría estar relacionada con la variabilidad de las cepas usadas (Mengeling *et al.,* 1996; Prieto *et al.,* 1997).

Probablemente, los embriones pueden infectarse durante las fases tempranas de la gestación; sin embargo, los cuadros clínicos más graves han sido descritos cuando la infección ocurre en el último tercio (Terpstra *et al.,* 1991; Chistianson *et al.,* 1992; Mengeling *et al.,* 1994; Chistianson *et al.,* 1993; Mengeling *et al.,* 1994; Lager *et al.,* 1996; Prieto *et al.,* 1996 y 1997; Kranker *et al.,* 1998).

El PRRSV puede provocar una infección persistente en los cerdos. La persistencia de la infección es independiente de la edad; puede ocurrir por una infección de los fetos *in utero*, o por una infección de animales jóvenes o adultos. Este estado se debe al acantonamiento del virus en pulmón y en diferentes órganos linfoides, sobre todo tonsilas, de duración variable pudiendo ser

detectado hasta 251 días post-infección (Fairbanks *et al*., 2002; Horter *et al*., 2002; Wills *et al*., 2003).

Tras la infección de las cerdas, algunos estudios han descrito la presencia del virus en los folículos ováricos (Swenson *et al.,* 1995; Prieto *et al.,* 1996 y 1997), pero otros no (Benson *et al.,* 2001). La posible presencia del virus en esta localización estaría asociada a la presencia de macrófagos (Sur *et al.,* 2001). Aparte de la diferencia de resultados, el efecto que el PRRSV pueda tener en el ovario y en el ciclo estral es aún desconocido, particularmente porque no ha podido demostrase su presencia en el óvulo y no se han hallado diferencias destacables entre el desarrollo de los folículos de cerdas infectadas y no infectadas (Sur *et al.,* 2001).

En el caso de los verracos, el PRRSV puede excretarse por el semen incluso en ausencia de viremia (Christopher-Hennings *et al.,* 1995 y 2001). Existe controversia sobre cómo el virus llega al semen; podría hacerlo por una diseminación directa a través de la sangre durante el periodo de viremia y replicarse en el tracto reproductor o bien por la migración a través de la sangre o de los conductos linfáticos de las células infectadas de la línea monocito/macrófago. Esta última vía, que no implicaría necesariamente la multiplicación del virus en las células del tracto reproductor (Christopher-Hennings *et al.,* 1998; Prieto *et al.,* 2003), parece la más probable. El virus se localiza en zonas del epidídimo ricas en macrófagos (Prieto *et al.,* 2003), puede aislarse de eyaculados de cerdos vasectomizados (Christopher-Hennings *et al.,* 1998) y prácticamente hay una ausencia de replicación en los testículos (Prieto *et al.,* 2003). Todos estos hallazgos explicarían porqué la excreción del virus a través del semen puede ser intermitente, ya que la presencia del virus en el aparato reproductor del verraco estaría más estrechamente relacionada con una dinámica de población de monocitos/macrófagos infectados procedentes de otras zonas que con la posible replicación en las células espermatogénicas (Prieto y Castro, 2005). Por otro lado, otros autores sostienen que el PRRSV puede replicarse en las células germinales del epitelio de los túbulos seminíferos, así como en los macrófagos localizados en testículos y glándulas anexas (Sur *et al*., 1997).

En definitiva, la patogenia del PRRS está asociada a la infección de las células de la línea monocito/macrófago. De hecho, el virus se localiza en el citoplasma de los macrófagos de cualquier órgano (Benfield *et al*., 1992; Rossow *et al*., 1995 y 1996), pero presenta especial tropismo por los del pulmón y los órganos linfoides, provocando neumonía intersticial y linfoadenopatía (Rossow *et al*., 1995 y 1996; Zimmerman et al., 2006). La infección no afecta a

todos los macrófagos. Se sabe que menos del 2% de los macrófagos son permisivos a la replicación del virus (Duan *et al*., 1997) y que el virus posee una especial predilección por los macrófagos inmaduros (Choi *et al*., 1994; Mengeling *et al*., 1995). Por otro lado, los macrófagos permisivos a la infección disminuyen con la edad (Thanawongnuwech *et al*., 1998). En consecuencia, todos estos factores explicarían el motivo por el que la enfermedad es más grave en cerdos jóvenes (Goyal, 1993). La secuencia de todos los fenómenos ocurridos durante la infección se resumen brevemente en la figura 3.

Figura 3. Patogenia del PRRSV (modificado a partir de K.D. Rossow, South Dakota State University).

La entrada del virus en los macrófagos podría llevarse a cabo mediante la unión del heterodímero formado por la GP5 y la proteína M con los sialoglicanos de la superficie de la célula y posterior endocitosis (Delputte *et al*., 2002). Sin embargo, la transfección del gen de la sialoadhesina en células no macrofágicas no es suficiente para permitir la replicación; por tanto, en este proceso intervienen otros fenómenos que hasta ahora se desconocen (Vanderheijden *et al.,* 2003). Además de la variabilidad individual, algunos estudios han demostrado una variabilidad específica de razas (Halbur *et al.,* 1998; Henryon *et al.,* 2001). Recientemente, Vincent *et al.* (2005 y 2006) han sugerido que las diferencias en la susceptibilidad al virus entre razas está probablemente relacionada con las características de las poblaciones celulares de la línea monocito/macrófago de cada raza.

La existencia de anticuerpos no neutralizantes puede incrementar la capacidad de infección del PRRSV. Este fenómeno no es necesario para la infección de los macrófagos pero se considera como una segunda vía de entrada a estos. Algunos autores han asociado este fenómeno, conocido como incremento dependiente de anticuerpos (o ADE acrónimo correspondiente a *antibody-dependent enhancement*), con el nivel y duración de la viremia (Yoon *et al.,* 1996; Shibata *et al.,* 1998).

Se ha descrito una elevada variabilidad de la virulencia entre cepas tanto a nivel sistémico como a nivel del aparato reproductor (Halbur *et al.,* 1996; Mengeling *et al.,* 1996; Park *et al.,* 1996; Thanawongnuwech *et al.,* 1998) Sin embargo, poco se conoce sobre los factores de virulencia de este virus. Basándose en la secuenciación de cepas virulentas y de las cepas derivadas tras la atenuación en múltiples pases celulares, algunos autores han observado que mutaciones en las ORF 1a, ORF 1b y ORF 6 podrían estar involucradas en los procesos de replicación y virulencia del PRRSV (Madsen *et al*., 1998; Nielsen *et al*., 2001; Yuan *et al*., 2001; Grebennikova *et al*., 2004).

Debido a que la GP5 de todas las cepas del PRRSV son muy similares en tamaño, estructura y secuencia al LDV (Plagemann, 1996), algunos de los fenómenos observados en este virus podrían extrapolarse al PRRSV. La habilidad del LDV para establecer infecciones persistentes y menos virulentas en el ratón podría estar correlacionada con el nivel de glicosilación de la GP5 (Chen *et al.,* 1998; Li *et al*., 1998). Sin embargo, los niveles de glicosilación de esta proteína en el PRRSV parece estar más asociada a fenómenos de escape de la respuesta inmune que a factores de virulencia (Ostrowski *et al*., 2002).

Algunos autores han demostrado que el PRRSV es un inductor de la apoptosis de macrófagos. Suárez *et al*. (1996) han sugerido que la GP5 está involucrada en este proceso. Sin embargo, muchos estudios han puesto de relieve que este fenómeno no se debe a un efecto directo de la replicación del virus, ya que la mayoría de los macrófagos apoptóticos no están infectados (Sirinarumitr *et al*., 1998; Sur *et al*., 1998), sino que podría deberse a la acción de citoquinas u otras moléculas liberadas en el transcurso de la infección (Choi y Chae, 2002; Labarque *et al*., 2003; Miller y Fox, 2004).

1.4 EPIDEMIOLOGÍA DEL PRRS

1.4.1 Distribución geográfica

Desde la aparición de los primeros brotes, la enfermedad se extendió rápidamente por América del Norte y por Europa. Se han descrito además infecciones por el genotipo europeo en EEUU (Dewey *et al*., 2000; Ropp *et al*., 2004) y por el genotipo americano en Europa (Botner *et al.,* 1997; Madsen *et al.,* 1998; Mortensen et al., 2002). Respecto al resto de los continentes, recientemente se ha descrito que ya en 1987, meses después de los casos de Carolina del Norte, el síndrome fue observado en Japón (Yoshii *et al.,* 2005) y en 1989 en Tailandia (Thanawongnuwech *et al.,* 2004). En la actualidad el PRRS se encuentra prácticamente en todos los países productores de porcino, de forma endémica en la mayoría de ellos (Zimmerman *et al*., 2003b).

Los cambios que se han considerado como "facilitadores" de la diseminación y perpetuación del virus en la cabaña porcina mundial son: el aumento del tamaño de las granjas ligado a la creación de zonas de alta densidad porcina, el aumento del transporte de cerdos vivos entre y dentro de un país, el aumento del uso de la inseminación artificial y el comercio internacional (Plana-Durán *et al*., 1992a; Shin *et al*., 1993; Dewey *et al*., 2000).

No existen estimaciones fiables sobre la prevalencia de la infección. Además, el uso a nivel mundial de vacunas vivas atenuadas no permite diferenciar a los cerdos vacunados de los infectados. Por otra parte, los virus atenuados vacunales pueden transmitirse entre animales (Botner *et al.,* 1997) por lo que resulta muy complicada esta estimación. Sin embargo, la prevalencia de la enfermedad en los mayores países productores se presupone elevada. De los

datos parciales que se han publicado, se considera que, si no ha existido un plan de control efectivo, la infección se encuentra presente en más del 70% de las granjas y en más del 50% de los cerdos (Hirose *et al*., 1995; Lu *et al*., 1999). Concretamente, en Europa se cree que las granjas están infectadas de forma endémica con valores superiores al 50% en cerdas reproductoras y prácticamente la totalidad de los cerdos de engorde (Geue, 1995; Maes, 1997; Nodelijk *et al.,* 2003).

1.4.2 Vías de transmisión y excreción

Se ha comprobado que los cerdos pueden infectarse por el PRRSV a través de numerosas vías de transmisión, incluyendo las vías intranasal, oral e intramuscular (Magar *et al*., 1995; Van der Linden *et al.,* 2003; Magar y Larochelle, 2004), intravenosa, intraperiotenal y vaginal (Yaeger *et al.,* 1993; Gradil *et al.,* 1996; Benfield *et al.,* 2000) e intrauterina (Terpstra *et al.,* 1991; Plana *et al.,* 1992a; Chistianson *et al.,* 1993).

Como norma general, la transmisión es totalmente dependiente de la vía de exposición y de la dosis del virus (Hermann *et al.,* 2005), así como de la edad; los animales jóvenes son más receptivos a la infección y secretan el virus durante más tiempo (Van der Linden *et al.,* 2003). Usando cerdos jóvenes, Hermann *et al.* (2005) evaluaron mediante curvas de dosis-respuesta varias vías de exposición al virus. La dosis infectiva$_{50}$ para la vía oral fue superior que para la intranasal (dosis infectiva de cultivo celular$_{50}$ $10^{5,3}$ versus $10^{4,0}$, respectivamente). Según estos autores, los cerdos son extremadamente receptivos a la infección por vía parenteral, más que por el resto de las vías analizadas hasta el momento. Por tanto, las prácticas realizadas en la manipulación habitual de los cerdos en la granja, crotado en orejas, cortes de colas y colmillos o la inoculación de medicamentos, así como las mordeduras y otras lesiones provocadas por la interacción entre cerdos, facilitaría la entrada del virus al comprometer todas estas situaciones la integridad de la piel. En consecuencia, la transmisión entre cerdos suele ocurrir por contacto directo entre animal infectado y animal susceptible, aunque también puede ocurrir por la exposición a fluidos contaminados o fómites. En relación a estos últimos, las agujas serían una de las principales fuentes de infección (Otake *et al*., 2002a). Los vehículos de transporte de animales también podrían actuar como una fuente importante (Dee *et al*., 2002, 2003, 2004 y 2005).

Otra forma de transmisión posible es a través de mosquitos y moscas que actuarían de vectores mecánicos (Otake *et al*., 2002b y 2003). La transmisión vía aerógena parece no ser un factor muy importante en la diseminación del virus ya que este se inactiva rápidamente. Experimentalmente esta vía sólo ha podido ser efectiva a distancias inferiores de 5 metros (Torremorell *et al*., 1997; Wills *et al*., 1997a; Kristensen *et al*., 2002). Pero existe cierta controversia, ya que algunos autores han podido demostrar el aislamiento de virus infectivo en aire recogido a 150 metros de distancia de una aerosolización experimental (Dee *et al*., 2005).

Respecto a la participación de otras especies como hospedadores del virus, hasta el momento sólo existe la descripción de alguna especie aviar. Zimmerman *et al.* (1997) demostraron que los patos reales son susceptibles al PRRSV, ya que se infectaron por la ingestión de agua contaminada y se pudo recuperar el virus de sus heces 39 días después de la exposición. Además, pudieron demostrar la transmisión del PRRSV entre dos individuos de esta especie y entre estas aves y los cerdos. Sin embargo, estudios más recientes (Trincado *et al.,* 2004 y Lager y Osorio, comunicación personal) no han podido demostrar la susceptibilidad de esta especie a la infección por el PRRSV.

La transmisión vertical es muy importante. El PRRSV es capaz de atravesar la barrera placentaria, principalmente durante el último tercio de la gestación, y por tanto infectar los lechones en el útero; pudiendo provocar en ocasiones el nacimiento de animales virémicos y asintomáticos (Christianson *et al*., 1993; Benfield *et al*., 1997; Lager *et al*., 1997a; Kranker *et al*., 1998), que infectarán a otros cerdos susceptibles en las posteriores fases de producción.

En los animales infectados la excreción del virus puede llevarse a cabo por la saliva, las secreciones nasales, el semen, la orina o la leche y el calostro (Robertson, 1992; Yaeger *et al*., 1993; Swenson et al., 1994; Christopher-Hennings *et al.*, 1995; Wills *et al.,* 1997b) y quizás por las heces (Rossow *et al.,* 1994; Wills *et al.,* 1997b). El período de excreción es muy variable, oscilando entre 9 y 92 días post-infección según la vía de excreción analizada (Rossow *et al*., 1994; Christopher-Hennings *et al*, 1995; Shin *et al.,* 1997; Wills *et al*., 1997b; Wagstrom *et al*., 2001; Christopher-Hennings *et al.*, 2001; Prieto *et al.,* 2003). La excreción por el semen puede ser intermitente y tener una elevada variabilidad entre individuos (Prieto y Castro, 2005). En ocasiones el virus puede hallarse en algunos fluidos, como la saliva o las muestras orofaríngeas, hasta 5 meses después de la infección (Wills *et al.,* 1997c). Según estos autores, este hecho estaría estrechamente relacionado con la persistencia del virus en las tonsilas. Estos hallazgos

sugieren de nuevo que el comportamiento de dominancia entre los cerdos desempeñaría un papel muy importante en la transmisión y en la perpetuación de la infección en la granja (Wills *et al.,* 1997b).

1.4.3 Incidencia clínica y factores de riesgo

El PRRSV puede afectar a cerdos de todas las edades. La morbilidad y la letalidad asociadas a esta infección son muy variables (de Jong *et al*., 1991; Done y Patton, 1995; Done *et al*., 1996; Le Potier *et al*., 1997) y están fuertemente relacionadas con la virulencia de cada cepa, las diferencias individuales de cada cerdo (estatus inmunitario y predisposición genética), edad o estado de gestación en el momento de la infección, estado general de la granja (recirculación de una misma cepa, entrada a una granja libre o entrada de una nueva cepa a una granja previamente infectada/inmunizada), y a los factores de ambiente y de manejo (Zimmermann *et al.,* 2003b y 2006). En un brote agudo de PRRS atípico la letalidad puede ser superior al 50% en lechones y al 10% en adultos (Rossow *et al.,* 1997). Estos valores son marcadamente inferiores en granjas endémicas, donde en ocasiones la infección puede pasar inadvertida (Nodelijk *et al.,* 2000 y 2003). Factores ambientales como la alta densidad dentro de la granja, el uso de sistemas de producción de flujo continuo y un gran tamaño de la explotación repercutirían negativamente en la incidencia de la enfermedad (Nodelijk *et al.,* 2000 y 2003).

La recirculación del virus en una granja puede llegar a ser indefinida gracias al flujo de animales susceptibles y a la presencia de animales infectados durante un largo periodo de tiempo o incluso persistentemente infectados (Dee y Joo, 1994; Nodelijk *et al.,* 2003). La transmisión del virus a cerdos susceptibles ha sido demostrada hasta 2 meses después de la infección experimental (Terpstra *et al.,* 1992). Los cerdos susceptibles pueden proceder de la reposición o del nacimiento de lechones de cerdas no infectadas. Además la irrupción de la infección en una granja no tiene porque provocar necesariamente la infección de todos los animales, de modo que siempre quedarán animales susceptibles de infectarse en cualquier momento (Terpstra *et al.,* 1992; Albina *et al.,* 1994; Nodelijk *et al.,* 2000). Algunas estimaciones predicen que una granja de tamaño medio mantiene la infección durante 10 veces menos tiempo que una granja del doble de tamaño (Nodelijk *et al.,* 2000).

1.5 CUADRO CLÍNICO Y LESIONAL

1.5.1 Generalidades

La infección del PRRSV ocurre principalmente en los macrófagos de todo el organismo, por lo que el resultado es una enfermedad sistémica. La infección de los macrófagos alveolares e intravasculares del pulmón provoca un cuadro respiratorio. La transmisión transplacentaria y la enfermedad sistémica de las cerdas son las causantes de la enfermedad reproductiva. Existe una marcada variabilidad de la sintomatología dependiendo principalmente del estado inmunitario del animal, de la edad y de la cepa infectante.

1.5.2 Animales adultos

Los signos clínicos más frecuentemente observados en animales adultos incluyen anorexia transitoria, fiebre moderada y letargia (Keffaber, 1989; Loula, 1991). De forma más ocasional se han descrito edemas subcutáneos y cianosis en orejas, extremidades y vulva (Hopper *et al*., 1992; Rossow, 1998). En algunas ocasiones la infección en animales adultos, especialmente en cerdas cerca del parto, puede ser fatal (Loula, 1991; Hopper *et al.,* 1992)

En los verracos, la infección puede provocar un descenso de la libido durante una semana (Hopper *et al.,* 1992; Feitsma *et al*., 1992). Existe controversia sobre el efecto de la infección en la calidad del semen (Prieto y Castro, 2005). En algunas ocasiones se han descrito alteraciones en la calidad del semen, como descenso en la motilidad de los espermatozoides y del porcentaje de espermatozoides con el acrosoma normal e incremento de las anomalías morfológicas (De Jong *et al.,* 1991; Feitsma *et al.,* 1992; Prieto *et al.,* 1996). Sin embargo, otros autores no han podido demostrar alteración alguna (Yaeger *et al.,* 1993; Swenson *et al.,* 1994).

Los primeros síntomas observados en las cerdas gestantes son anorexia y fiebre y en ocasiones pueden pasar inadvertidos. En una segunda fase de la infección aparecen los fallos reproductivos. Se han descrito incrementos en el tiempo de retorno al celo, descenso en la intensidad de los celos e incluso abortos tempranos (Keffaber, 1989; Loula, 1991; Lager *et al.,* 1996). Inicialmente se asociaron los problemas reproductivos de la infección con cerdas infectadas en estado avanzado de gestación (último tercio) (Cromwijk, 1991; Hill., 1990), pero también se ha podido observar que la infección en estados tempranos provoca problemas

reproductivos (Terpstra *et al.,* 1991; Chistianson *et al.,* 1992; Plana *et al.,* 1992a; Mengeling *et al.,* 1994). Si la infección ocurre cerca del parto, generalmente la camada es una mezcla variable de abortos tardíos, fetos momificados, lechones nacidos muertos, lechones débiles y cerdos normales, infectados o no, con el consecuente aumento de la mortalidad en lactación (Christianson *et al*., 1991).

1.5.3 Lechones

Existe una marcada variabilidad, influyendo en gran medida la edad del animal (Rossow *et al*., 1994). Los lechones nacido vivos e infectados pueden presentar apatía, anorexia, disnea y cianosis (White, 1992, Rossow *et al*., 1999). La sintomatología típica en fases de producción más avanzadas es muy parecida pero más leve. Hay un aumento de colas de producción y en cerdos enfermos la ganancia media diaria disminuye en un 25%-50%.

Los animales infectados pueden presentar un cuadro respiratorio grave (Loula, 1991). Esta forma respiratoria de la enfermedad es más típica de granjas infectadas crónicamente, apareciendo de forma más marcada durante la fase de transición pero pudiéndose hallar también en la fase de cebo. Otros síntomas que ocasionalmente pueden observarse son edemas perioculares, conjuntivitis, cianosis en punta de orejas y eritema (Rossow, 1998).

1.5.4 Lesiones

Las lesiones macroscópicas son moderadas en todas las edades, aunque pueden ser algo más marcadas en los animales jóvenes. La distribución del virus es por todo el organismo, pero las lesiones tanto a nivel macroscópico como a nivel microscópico se centran en pulmón y órganos linfoides (Tabla 1 y figuras 4, 5 y 6).

	Lesiones Macroscópicas	Lesiones Microscópicas
Pulmón	Fallo colapso pulmonar Afectación variable (0-100%) Consolidación pulmonar Edema interlobular	Neumonía intersticial: Infiltración de septos alveolares de células mononucleares, hiperplasia e hipertrofia de pneumocitos tipo II y marcada acumulación de células inflamatorias y exudado necrótico alveolar
Linfonodos	Marcado agrandamiento y/o congestión: regiones cervical, torácica craneal e inguinal	Linfadenopatía con hipertrofia e hiperplasia de los centros germinales Focos de necrosis folicular
Fetos	Inconstantes y no características Hemorragia del cordón umbilical Edema perrirenal y mesentético	Arteritis necrotizante del cordón umbilical
Otros	Reproductoras: Edema endometrial	Reproductoras: Leve a moderada endometritis y miometritis Lechones: Meningoencefalitis, Miocarditis, Arteritis

Tabla 1. Lesiones macroscópicas y microscópicas asociadas a la infección del PRRSV.

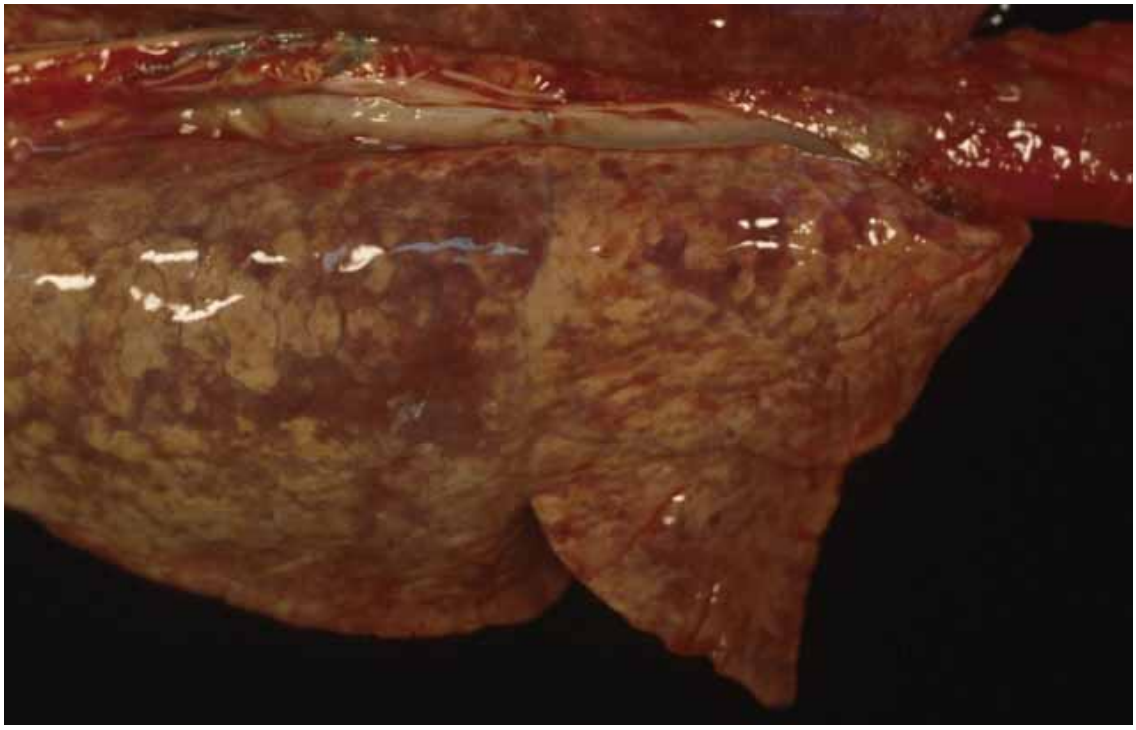

Figura 4. Pulmón: ausencia de colapso pulmonar con marcado patrón lobulillar.[2]

[2] Imágenes cortésmente cedidas por el Dr. J. Segalés (Universitat Autònoma de Barcelona-Centre de Recerca en Sanitat Animal)

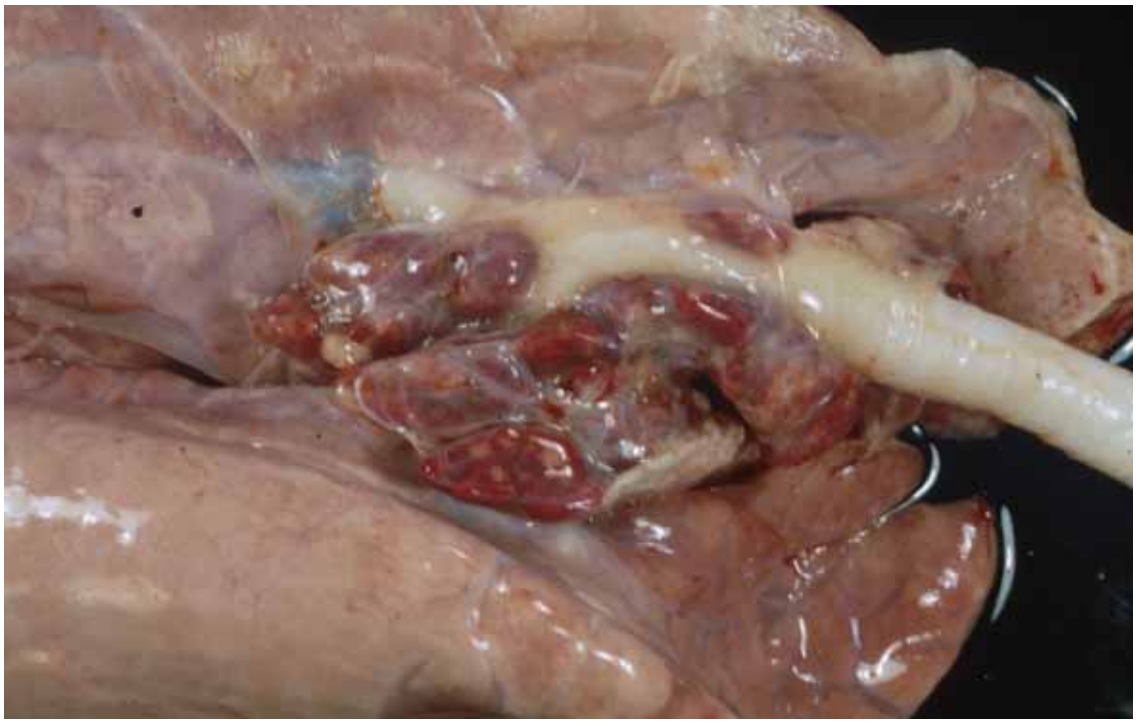

Figura 5. Moderado a marcado incremento de tamaño del linfonodo mediastínico.[2]

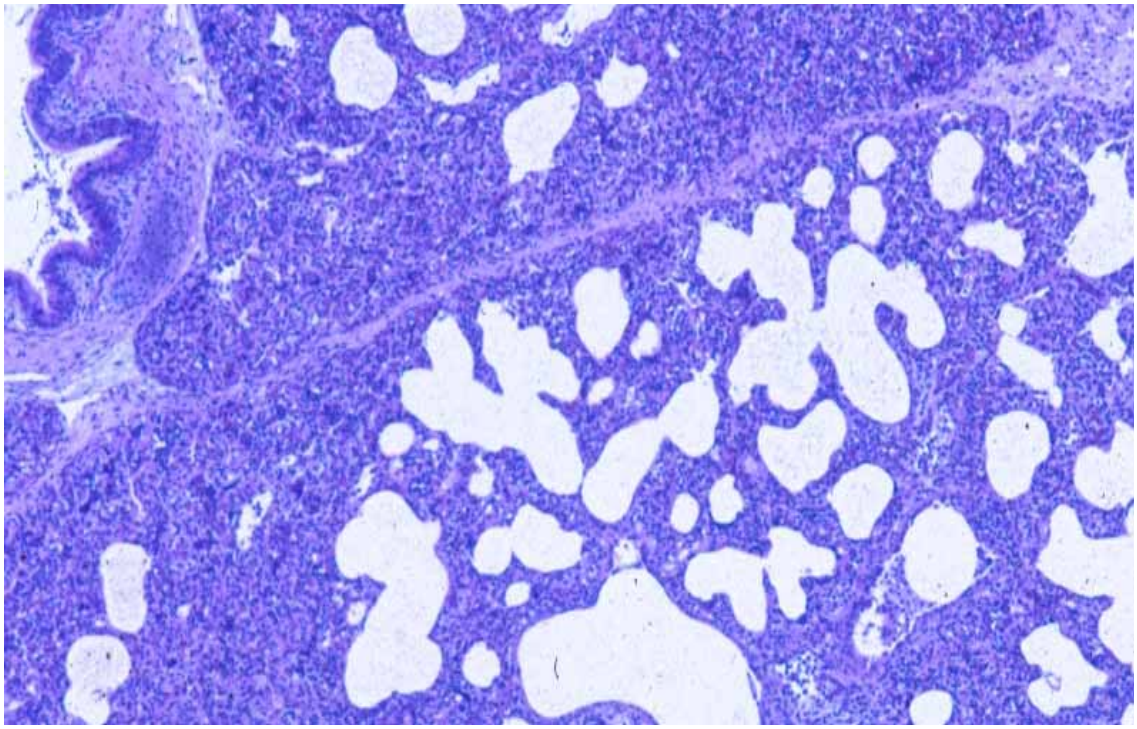

Figura 6. Pulmón: moderado incremento del grosor de los tabiques interlobulillares debido al acúmulo de células inflamatorias mononucleares (neumonía intersticial subaguda). Tinción de hematoxilina-eosina.[2]

[2] Imágenes cortésmente cedidas por el Dr. J. Segalés (Universitat Autònoma de Barcelona-Centre de Recerca en Sanitat Animal)

1.6 DIAGNÓSTICO

1.6.1 Generalidades

El diagnóstico clínico del PRRS puede ser complejo porque la sintomatología observada es compatible con muchas otras enfermedades. En una granja, un brote activo de PRRS provoca una serie de alteraciones en los parámetros productivos que en muchos casos no puede diferenciarse de una infección por parvovirus, virus de la enfermedad de Aujeszky o circovirus porcino tipo 2, entre otros virus (Halbur, 2003). Del mismo modo, el diagnóstico anatomopatológico puede ser confuso, ya que las lesiones provocadas por el PRRSV son muy poco evidentes, apareciendo además coinfecciones de forma frecuente. Por tanto es necesario llevar a cabo un diagnóstico de laboratorio. La confirmación puede efectuarse mediante la detección del virus, aislamiento o detección de los antígenos víricos o del ácido nucleico, o mediante la detección de anticuerpos.

1.6.2 Detección del virus

1.6.2.1 Aislamiento del virus

El aislamiento del virus se realiza en MAPs o sublíneas de la línea celular de riñón de mono MA-104 (CL-2621, CRL11171 y MARC-145). El efecto citopático del PRRSV es muy evidente en MAPS tras 3-5 días de incubación, pero en las líneas celulares es muy leve y requiere uno o más pases en cultivo, y generalmente para verificar el aislado es necesaria la aplicación de una técnica complementaria para la detección del virus, como la inmunofluorescencia (IF) o la prueba de inmunoperoxidasa en monocapa (o IPMA acrónimo correspondiente a i*mmunoperoxidase monolayer assay*). No todas las cepas tienen la misma capacidad de replicación en todos los tipos celulares; el uso de MAPs es necesario para la detección de la mayoría de cepas del genotipo europeo, por lo que se recomienda que al menos en estos casos sean usados dos tipos de cultivos celulares (Dewey *et al.,* 2000; Yoon *et al.,* 2003).

En lechones, durante el primer mes de la infección, las mejores muestras para el aislamiento son el suero, pulmón, linfonodos y tonsilas, pero en infecciones persistentes, las tonsilas, los linfonodos y los raspados orofaríngeos son más aconsejables que el suero o el pulmón. El

aislamiento del virus no suele realizarse de rutina en los laboratorios de diagnóstico clínico ya que conlleva la obtención de MAPs o el mantenimiento de líneas celulares específicas.

1.6.2.2 Detección de los antígenos víricos

Las técnicas más usadas para la detección de antígenos del PRRSV son la IF (Benfield *et al.,* 1992) y la inmunohistoquímica (Van Alstine *et al.,* 2002). La IF es más rápida y económica, pero la inmunohistoquímica es más sensible; en ambas la lectura debe ser cuidadosa para evitar falsos positivos. Ambas técnicas usan los mismos anticuerpos monoclonales (SDOW17 o SR30) para detectar la nucleocápside del virus en el citoplasma de la célula infectada.

1.6.2.3 Detección del ácido nucleico vírico

En la actualidad, para la detección del ácido nucleico del virus, se usan principalmente la técnica de hibridación *in situ* (detección en cortes de tejidos) (Sur *et al.,* 1996; Haynes *et al.,* 1997) y la transcripción inversa de la reacción en cadena de la polimerasa (o RT-PCR acrónimo correspondiente a *reverse transcription-polymerase chain reaction*) y sus variantes (RT-PCR anidada y RT-PCR cuantitativa). Los ensayos basados en la reacción en cadena de la polimerasa son los que en general tienen una sensibilidad y una especificad mayor. Para este último grupo de técnicas se pueden usar distintas muestras: suero, semen, raspados orofaríngeos, lavados traqueobronquiales o homogenizados de tejidos, por ejemplo pulmón. También se pueden usar como muestras algunos tejidos o secreciones que son tóxicos para los cultivos celulares, como el semen o las heces. Las viremias son largas y variables, por tanto los periodos de detección mediante la amplificación del genoma del virus a partir del suero son extensos pero con una marcada variabilidad. Sin embargo, se puede detectar el virus durante largos periodos a partir de homogenizados de tonsila (Wills *et al.,* 2003).

La detección del ácido nucleico vírico no se relaciona directamente con la presencia de virus infectivo, pero se ha hallado que la correlación entre la RT-PCR y aislamiento del virus bajo condiciones experimentales es superior al 80% (Christopher-Henninng *et al.,* 1995; Horter *et al.,* 2002). Por otro lado, la RT-PCR puede perder sensibilidad cuando se usan mezclas de muestras, un hecho muy común en la rutina del laboratorio clínico. Incluso con muestras individuales a veces es necesario el uso de la RT-PCR anidada para aumentar la sensibilidad. Además, según el diseño de los *primers* y los fragmentos a detectar, la variabilidad genética del

PRRSV puede ser un inconveniente. Para solventar este problema se ha propuesto la detección de las ORFs más constantes (ORF 6 y 7). El uso de sistemas automatizados de RT-PCR a tiempo real ayuda a estandarizar los resultados entre laboratorios (Wasilk *et al.,* 2004), ya que hasta ahora cada laboratorio ha creado y trabajado con sus propios *primers.* Algunas RT-PCR han sido diseñadas para diferenciar entre cepas del genotipo europeo y del americano (Gilbert *et al.,* 1997), pero hasta la fecha la RT-PCR no permite diferenciar entre cepas vacunales y cepas salvajes ya que no existen cepas vacunales con una delección apropiada para este fin. Este problema puede solventarse mediante la secuenciación del amplicón resultante de la RT-PCR (Christopher-Henninng *et al.,* 2002; Roberts, 2001 y 2003). Si el objetivo de la detección del ácido nucleico es analizar los cambios ocurridos entre cepas o en una misma cepa en una población, la ORF 5 es una buena diana para la amplificación y secuenciación. En estos casos, se aconseja amplificar también la ORF 6 como control por ser una secuencia muy conservada. La secuenciación de las cepas también es útil para monitorizar el movimiento, el origen y la evolución de las cepas de una granja o población (Murtaugh *et al.,* 1995; Andreyev *et al.,* 1997; Pirzadeh *et al.,* 1998; Nelsen *et al.,* 1999; Meng, 2000; Mateu *et al.,* 2006).

1.6.3 Detección de la respuesta humoral

1.6.3.1 Anticuerpos totales

En Europa, las técnicas más usadas para la detección de anticuerpos en suero son el IPMA, el ELISA y la seroneutralización. La detección de anticuerpos mediante el IPMA requiere un cultivo de MAPs o de una línea celular continua que soporte la replicación del virus, por lo que es más compleja que el ELISA. Esta última técnica, junto a la RT-PCR, es la técnica más extendida como diagnóstico de campo. Según el tipo de ELISA se puede diferenciar entre infecciones por el genotipo europeo o americano. Los ELISAs comerciales están generalmente dirigidos hacia la proteína N. Una recomendación que debe hacerse a la hora de interpretar los resultados es que un resultado positivo no puede relacionarse directamente con una infección reciente. Los anticuerpos pueden detectarse durante un periodo de tiempo muy largo; a partir de los 7-14 días post-infección y desaparecen generalmente antes de los 12 meses, aunque se han detectado hasta los 600 días (Nelson *et al*., 1994; Vezina *et al*., 1996; Labarque *et al*., 2000; Desrosiers y Boutin, 2002; Meier *et al*., 2003). Además, los anticuerpos maternales duran de media hasta las cuatro o cinco semanas de edad, por lo que es habitual su detección en lechones en maternidad o al inicio de la fase de transición (Nodelijk *et al.,* 1997; Melnichouk *et al.,* en prensa). Por otro lado, un resultado negativo puede darse en un animal persistentemente infectado pero que ha

seronegativizado (Wills *et al.,* 2003). Recientemente, se ha demostrado una muy elevada variabilidad de sensibilidad y especificidad de diferentes ELISAs comerciales y, por tanto en la aparición de falsos positivos y falsos negativos (Mielli *et al.* 2002; Yoon *et al.,* 2003). Se ha constatado un porcentaje de falsos positivos en torno al 0.5%-2% de las muestras (Keay *et al.,* 2002; O´Connor *et al.,* 2002; Ferrin *et al.,* 2004). Analizando tres ELISAs comerciales se ha observado que el valor de coincidencia (Kappa) no supera el 0.63 (Mateu *et al.,* en prensa). En este estudio se analizaron varias granjas, y aunque todos los ELISA detectaron la mayoría de granjas infectadas como tal, el porcentaje de animales detectados en cada granja fue distinto. Además, uno de los ELISA clasificó una granja infectada como negativa. Por tanto, las propiedades inherentes de cada ELISA, principalmente por los antígenos usados en el tapizado de la placa, determinaría una sensibilidad diferente para cada uno. La elevada prevalencia del PRRSV, junto a todos estos inconvenientes, aconseja no usar un único resultado positivo como explicación en una granja a un hallazgo clínico compatible con PRRS.

Otra de las técnicas descritas en la bibliografía es la inmunofluorescencia indirecta (IFI). Esta técnica permite la detección diferenciada de Inmunoglobulinas M y de Inmunoglobulinas G. Las primeras se detectan desde los 5 hasta los 28 días post-infección y las segundas desde los 10 hasta los 120 días post-infección (Joo et al., 1997; Zhou et al., 2002). La sensibilidad de esta técnica está muy asociada a la diferencia antigénica entre la cepa usada en la IFI y la cepa del PRRSV que indujo los anticuerpos a detectar en la muestra problema; por ejemplo, prácticamente no existe reacción cruzada entre cepas del genotipo americano y cepas del genotipo europeo mediante IFI (Christopher-Hennings *et al.,* 2002).

1.6.3.2 Neutralización vírica

La detección de los anticuerpos neutralizantes (AN) se considera una prueba con una elevada especificidad, pero su aplicación en la valoración de la respuesta inmune o en el diagnóstico tiene el inconveniente de detectar anticuerpos que no aparecen hasta varias semanas después del inicio de la infección/vacunación. Por otro lado, los resultados de la técnica pueden variar según el grado de homología entre el virus usado en la técnica y la cepa problema (Ansari *et al.,* 2006). Añadiendo complemento al suero que va a ser analizado, mediante la adicción de suero fresco porcino o suero de cobayo, puede mejorarse la detección temprana de los AN en presencia de Inmunoglobulinas M (Yoon *et al.,* 1994; Takikawa *et al.,* 1997). Esta técnica es de uso experimental y no suele usarse en un laboratorio de diagnóstico.

1.7 ASPECTOS INMUNOLÓGICOS EN LA INFECCIÓN POR EL PRRSV

1.7.1 Generalidades

Desde las primeras investigaciones al respecto, se observó que la respuesta inmune del cerdo frente al PRRSV no era completamente eficaz. La existencia de animales con viremias largas o infecciones persistentes (Wills *et al.,* 1995 y 1997c) y la ineficacia parcial o total frente a re-infecciones heterólogas (van Woensel *et al.,* 1998; Meng, 2000) evidencian un déficit en la respuesta. Los estudios inmunológicos, realizados principalmente con cepas del genotipo americano, han demostrado que los parámetros que podrían estar relacionados con una protección efectiva no alcanzan niveles óptimos hasta bastante tiempo después del inicio de la infección (Meier *et al.,* 2003; Osorio y López, 2004; Royaee *et al.,* 2004; Lowe *et al.,* 2005).

Un segundo factor que contribuye a la capacidad del PRRSV para escapar del control inmune es su diversidad genética (Meng, 2000). Diferentes estudios han mostrado que, tras una infección, se desarrolla una protección casi completa frente a re-infecciones homólogas (Lager *et al.,* 1997a, 1997b y 1999), pero esta protección es parcial o inexistente frente a re-infecciones heterólogas (Lager *et al.,* 1999; Labarque *et al.,* 2004). Delputte *et al.* (2004) han confirmado que la variabilidad genética dentro de un mismo genotipo tiene un claro reflejo en los antígenos reconocibles por el sistema inmune, ya que los AN creados frente a una cepa muestran una menor afinidad frente a otras cepas. Así mismo, Labarque *et al.* (2004) han mostrado que la variabilidad genética afecta también a la eficacia de las vacunas.

1.7.2 Respuesta innata frente al PRRSV

La respuesta innata constituye una primera barrera de lucha frente a una infección. También es de vital importancia para el desarrollo de la respuesta adquirida, ya que crea el ambiente adecuado que va a estimular y a facilitar la funcionalidad de las células participantes. En el transcurso de una infección por el PRRSV la respuesta innata es débil, en términos de la liberación de citoquinas (Van Reeth *et al.,* 1999 y 2002; Royaee *et al.,* 2004) y de la acción citotóxica de las células NK (Samson *et al.,* 2000; Lamontagne *et al.,* 2003).

Una de las citoquinas que desempeñan un papel más importante en la respuesta frente a una infección vírica es el interferón-α (IFN-α). Esta citoquina está considerada como un marcador de

la respuesta innata antivírica, ya que induce la producción de proteínas que participan en la supresión de la síntesis de proteínas víricas y condiciona un estado antivírico en las células vecinas aún no infectadas. Varios estudios han mostrado que el IFN-α podría ser crucial en el control de la infección *in vitro* del PRRSV, al inhibir la replicación del virus en los cultivos celulares (Albina *et al*., 1998a; Buddaert *et al*., 1998). Sin embargo, los estudios *ex vivo* muestran que la expresión de esta citoquina tras la infección por el PRRSV es extremadamente baja (Albina *et al*., 1998a; Buddaert *et al*., 1998; Royaee *et al*., 2004) y puede situarse en magnitudes del orden de 1.000 veces inferiores a las observadas tras otras infecciones víricas respiratorias del cerdo, como el coronavirus respiratorio porcino o el virus de la influenza (Van Reeth *et al*., 1999). Se desconoce el mecanismo por el cual el PRRSV inhibe o no estimula la producción de IFN-α, pero se sabe que este fenómeno es dependiente de la cepa que se examine (Lee *et al.,* 2004) y que desaparece si el virus se inactiva (Albina *et al.,* 1998a).

Otras citoquinas proinflamatorias de vital importancia para el correcto desarrollo de la respuesta innata también presentan un patrón de producción atípico frente a este virus. Numerosos estudios han demostrado que la liberación de TNF-α como respuesta al PRRSV es prácticamente inexistente (van Reeth *et al.,* 1999; Chiou *et al.,* 2000; López-Fuertes *et al.,* 2000; Thanawongnuwech *et al.,* 2001). Por el contrario, Van Reeth *et al.* (1999) observaron una elevada producción de IL-1 tras la infección. Según estos autores, la liberación de IL-1 estaría estrechamente relacionada con la masiva infiltración de células mononucleares en el pulmón (Paton *et al.,* 1992; Collins *et al.,* 1992; Nielsen y Botner 1997; Shibata *et al.,* 1997) y con los síntomas más comúnmente observados tras la infección del PRRSV: fiebre y anorexia (Keffaber, 1989; Loula, 1991).

Respecto a la respuesta citotóxica natural, en las primeras horas post-infección no se observa un aumento significativo de las células NK, ni en sangre ni en tejidos (Lamontagne *et al*., 2003). Samsom *et al.* (2000) sólo pudieron observar un aumento de esta población celular cinco días después de la infección. Según Murtaugh *et al.* (2002) este fenómeno es totalmente coherente con la débil respuesta de IFN-α observada, ya que esta citoquina es un potente activador y estimulador de la proliferación de las células NK.

En consecuencia, se considera que uno de los factores que determinan la existencia de una respuesta adquirida humoral y celular subóptima frente al PRRSV es la baja estimulación de la respuesta innata (Murtaugh *et al.,* 2002; Royaee *et al.,* 2004; Xiao *et al.,* 2004).

1.7.3 Respuesta adquirida frente al PRRSV

1.7.3.1 Respuesta humoral

Los anticuerpos específicos frente al PRRSV aparecen rápidamente tras la infección y en la mayoría de animales pueden detectarse ya en los primeros 10-14 días post-infección mediante ELISA o IPMA (Nelson *et al.,* 1994; Vezina *et al.,* 1996; Labarque *et al.,* 2000; Meier *et al.,* 2003). Desrosiers y Boutin (2002) observaron la presencia de anticuerpos en cerdas reproductoras hasta 2 años después de la infección.

La mayoría de estos anticuerpos están dirigidos hacia la proteína N, donde se encuentra el epítopo inmunodominante del virus (Yoon *et al*., 1995; Plana-Duran *et al*., 1997b), y no tienen la capacidad de neutralizar al virus. Esta incapacidad se ha demostrado tanto en estudios *in vitro* (Yoon *et al.,* 1994) como en experimentos de protección pasiva (López y Osorio, 2004). Por tanto, estos anticuerpos no desempeñarían ninguna función en la eliminación del PRRSV ni en la protección frente a re-infecciones (Meulenberg *et al*., 1995c; Labarque *et al.,* 2000; Murtaugh *et al*., 2002). También se han detectado anticuerpos no neutralizantes frente a la proteína no estructural 2 (nsp2), la GP4, la GP5 y la proteína M (Muelenberg *et al.,* 1993; Magar *et al.,* 1997; Oleksiewicz *et al.,* 2001; Ostrowski *et al.,* 2002; Cancel-Tirado *et al.,* 2004).

Yoon *et al*. (1996) describió que el PRRSV está asociado al fenómeno ADE. Estos autores observaron que la presencia de anticuerpos no neutralizantes en cultivos de macrófagos infectados por el PRRSV podía aumentar la capacidad de infección del virus en los macrófagos permisivos. La unión de los complejos antígeno-anticuerpo con el receptor Fc del macrófago permitiría la entrada del virus en la célula a modo de caballo de Troya (Choi *et al*., 1992; Christianson *et al*., 1993; Yoon *et al*., 1996 y 1997). Por tanto, estos anticuerpos no participan en la neutralización del virus y su presencia podría ser contraproducente.

La aparición de los AN es mucho más tardía que la de los anticuerpos no neutralizantes. Los AN empiezan a detectarse a partir de las cuatro semanas desde el inicio de la infección (Yoon *et al*., 1994; Loemba *et al*., 1996; Albina *et al.,* 1998b; Meier *et al.,* 2000 y 2003) y pueden mantenerse durante meses, aunque generalmente lo hacen a niveles muy bajos (Yoon *et al.*, 1995; Loemba *et al.*, 1996; Albina *et al*., 1998b; Labarque *et al*., 2000). El principal epítopo de neutralización se encuentra en la GP5 (Pirzadeh y Dea, 1997 y 1998; Osorio y López, 2005; Wissink *et al.,* 2003),

pero los AN también pueden estar dirigidos hacia la GP4 y la proteína M (Weiland *et al.,* 1999; Yang *et al.,* 2000; Bastos *et al.,* 2004).

1.7.3.2 Respuesta celular

Tras la infección se ha descrito un periodo de leucopenia y linfopenia que desaparece en 8-10 días (Christianson *et al.,* 1993; Nielsen y Botner, 1997). Albina *et al.* (1998b) no observaron cambios significativos en estos parámetros, pero observaron que durante este periodo la respuesta de los linfocitos frente a un mitógeno decreció significativamente, lo que sugiere una disminución de su funcionalidad o una regulación negativa por parte del virus.

Después de esta primera fase de la infección existe un desequilibrio de origen incierto en los porcentajes de las poblaciones de linfocitos circulantes. Bautista y Molitor (1997) observaron un aumento de la respuesta específica asociada principalmente a la proliferación de linfocitos $CD4^+$ y, en un menor porcentaje, a la de linfocitos $CD8^+$. En estudios posteriores se determinó que la proliferación específica estaría dirigida hacia la GP5, la proteína M y la proteína N (Bautista *et al.,* 1999; Kwang *et al*., 1999). López-Fuertes *et al.* (1999) también observaron una proliferación específica de linfocitos $CD4^+$ que, en este caso, mostraban un patrón clásico de secreción de citoquinas Th1. Sin embargo, estos mismos autores observaron que la re-estimulación *in vitro* de las células mononucleares de sangre periférica (CMSP) con el virus provocaba una proliferación policlonal de linfocitos $CD4^+/CD8^+$, $CD8^+$ y γδ. Más recientemente Olin *et al.* (2005) han observado un aumento de linfocitos γδ circulantes entre los 14 y los 70 días después de la infección, sin que se conozca aun el papel que estos linfocitos desarrollan durante la respuesta inmune frente al PRRSV. Contrariamente a los fenómenos descritos anteriormente, otros estudios han evidenciado un aumento exclusivo de linfocitos $CD8^+$, que se da lugar en sangre, en pulmón y en órganos linfoides a partir de las tres semanas post-infección. (Shimizu *et al.,* 1996; Albina *et al.,* 1998b; Samsom *et al.,* 2000; Lamontagne *et al.,* 2003; Tingstedt y Nielsen, 2004).

Un modo de evaluar la respuesta celular específica es la medición de células productoras de IFN-γ (CP-IFN-γ) usando la técnica inmunoenzimática denominada ELISPOT (Czerkinsky *et al.,* 1988; Zuckermann *et al.,* 1998; Díaz y Mateu, 2004). La importancia del IFN-γ frente al PRRSV queda demostrada en estudios *in vitro* en los que el pre-tratamiento de macrófagos alveolares con esta citoquina inhibió la replicación vírica y disminuyó el número de macrófagos que

permitían la replicación (Bautista y Molitor, 1999; Rowland *et al*., 2001). A pesar de que el RNA mensajero del IFN-γ se ha detectado en linfonodos, pulmón y CMSP de animales infectados por el PRRSV (López-Fuertes *et al.,* 1999; Rowland *et al.,* 2001), los datos disponibles sugieren que la respuesta de IFN-γ es baja e insuficiente. Recientemente, los estudios realizados con cepas americanas han demostrado que en la respuesta celular frente al PRRSV, las frecuencias de CP-IFN-γ son muy bajas y con una evolución lenta e irregular (Meier *et al*., 2003).

Los fenómenos anteriormente descritos demuestran que, frente a las cepas americanas, la respuesta inmune eficiente, valorada como la producción de AN y de CP-IFN-γ específicas de virus, se desarrolla en una fase tardía de la infección (Meier *et al*., 2003). Algunos autores han sugerido que el retraso en la formación de la respuesta podría estar ligado a una estrategia de evasión del virus. Dicha estrategia se basaría en una alteración de la respuesta inmune en las fases tempranas, que provocaría la inhibición de la producción de IFN-α y/o IFN-γ (Albina *et al.,* 1998a; Rowland *et al.,* 2001; Royaee *et al.,* 2004).

Resumen de los hallazgos más significativos descritos durante la respuesta inmune del cerdo frente a la infección del PRRSV

- **Leve estimulación de la respuesta innata; nula o escasa liberación de citoquinas propias de la respuesta innata (Cepas del genotipo americano y europeo). (Albina *et al.,* 1998a; Van Reeth *et al.,* 1999; 2002; Royaee *et al.,* 2004).**
- **Rápida y elevada producción de anticuerpos no neutralizantes (Cepas del genotipo americano y europeo). (Vezina *et al.,* 1996; Labarque *et al.,* 2000).**
- **Lenta y escasa producción de anticuerpos neutralizantes. (Cepas del genotipo americano y europeo). (Yoon *et al.,* 1994; Loemba *et al.,* 1996; Meier *et al.,* 2000, 2003).**
- **Lento e irregular desarrollo de las frecuencias de células productoras de IFN-γ (Cepas del genotipo americano). (Meier *et al.,* 2003).**

1.8 VACUNAS

1.8.1 Generalidades

El gran impacto económico del PRRS en la producción porcina y su rápida expansión por todos los países productores, generó desde el inicio un elevado interés en la producción de vacunas que controlaran la enfermedad. Hasta la fecha las vacunas comercializadas son inactivadas o atenuadas. La primera vacuna inactivada (Cyblue®, Cyanamid) fue creada en España y apareció en el mercado en 1993, mientras que la primera vacuna atenuada se creó en EEUU en 1994 (Ingelvac PRRS®, Boehringer Ingelheim). El uso tanto de vacunas inactivadas como de vacunas atenuadas está muy extendido, a pesar de que su efectividad es parcial (Mengeling *et al.,* 1999; Hurd *et al.,* 2001; Labarque *et al.,* 2004).

Uno de los principales problemas al que deben hacer frente ambos tipos de vacunas es la elevada variabilidad genética del virus, que compromete la protección frente a cepas heterólogas. Utilizando una vacuna atenuada (Porcilis®PRRS, Intervet), Labarque *et al*. (2004) demostraron la protección total frente a un desafío con la cepa de referencia europea (LV). La cepa vacunal usada y el LV comparten el 98% de homología en la ORF 5. Sin embargo, cuando los cerdos vacunados se desafiaban con una cepa italiana, que compartía con la cepa vacunal el 84% de homología en la ORF 5, se establecía viremia y el virus podía aislarse de los lavados traqueobronquiales. No obstante, puede considerarse que los cerdos vacunados tenían cierta protección heteróloga, ya que los títulos víricos detectados eran inferiores a los de los cerdos no vacunados e infectados. Otros estudios han concluido que las vacunas inactivadas tampoco aportan una respuesta eficaz frente a infecciones heterólogas. Nielsen *et al.* (1997) observaron que los verracos vacunados con una vacuna inactivada (Cyblue®, Cyanamid) y posteriormente infectados con una cepa de PRRSV danesa, presentaban los mismos niveles de viremia y de excreción del virus en semen que los verracos no vacunados e infectados.

Así pues, podemos afirmar que las vacunas otorgan una protección total frente a cepas homólogas pero parcial o inexistente frente a cepas heterólogas (Lager *et al.,* 1997a, 1997b y 1999; van Woensel *et al.,* 1998; Mengeling *et al.,* 1999 y 2003b; Labarque *et al.,* 2003 y 2004).

Existen otros dos problemas de difícil solución que deben afrontar las vacunas. Primero, la dificultad en el laboratorio de obtener una carga de antígeno suficiente. Obviamente este inconveniente lo es mucho más en el caso de las vacunas inactivadas, ya que no conservan la capacidad de replicación. El segundo inconveniente es que las células diana del PRRSV son las células del linaje monocito/macrófago, que son puntales en la coordinación de la respuesta inmune. En este caso, el inconveniente es mucho mayor para las vacunas atenuadas, ya que potencialmente aun conservan su poder infectivo.

1.8.2 Vacunas convencionales

1.8.2.1 Vacunas atenuadas

Se considera que las vacunas atenuadas son más eficaces que las inactivadas porque inducen una respuesta celular y humoral mayor (Roof *et al.,* 2000; Medvezcky *et al.,* 2002; Pesch *et al.,* 2005). A pesar de ello, las respuestas inmunes inducidas por las vacunas atenuadas parecen ser aun insuficientes para proteger de la infección (Gorcyca et al., 1995; van Woensel *et al.,* 1998; Meier *et al.,* 2003; Labarque *et al.,* 2004).

Las vacunas atenuadas inducen una producción baja de IFN-α (Sipos *et al.,* 2003; Royaee *et al.,* 2004), que como ya hemos visto anteriormente desempeña un papel muy importante en el desarrollo de la respuesta adquirida. Respecto a la respuesta celular adaptativa, las vacunas atenuadas del genotipo americano son capaces de inducir el desarrollo de CP-IFN-γ específicas de virus. Sin embargo, este desarrollo es muy inferior al obtenido con las vacunas atenuadas de otros virus comunes del cerdo. Por ejemplo, según Meier *et al.* (2003), tres semanas después de vacunar con la vacuna atenuada IngelvacPRRS®, la frecuencia media de CP-IFN-γ específicas de virus era de 82±25/10^6 CMSP. En comparación, tres semanas después de vacunar con una vacuna atenuada del virus de la enfermedad de Aujeszky, la frecuencia media de CP-IFN-γ era de 289±82/10^6 CMSP; es decir del orden de 2 a 3.5 veces superior al resultado obtenido con la vacuna de PRRSV. De hecho, en este estudio la frecuencia de CP-IFN-γ específicas de PRRSV no remontó hasta valores equiparables hasta varios meses después de la vacunación. Ni la revacunación ni la adición de un adyuvante convencional de aceite-en-agua mejoraron sustancialmente los resultados de la vacunación, mientras que sí lo hicieron con la vacuna de Aujeszky.

Respecto a la respuesta humoral, las vacunas atenuadas inducen una rápida producción de anticuerpos no neutralizantes detectables en todos los animales a partir de las 2 semanas tras la vacunación, descendiendo su producción a partir de las 32 semanas (Meier *et al.,* 2003). Por tanto, las vacunas atenuadas pueden estar potencialmente asociadas al ADE (Yoon *et al.,* 1997) Por otro lado, la aparición de los AN es muy irregular. Meier *et al.* (2003) sólo pudieron detectar AN en el 50% de los animales y nunca antes de trascurridas 8 semanas después de la revacunación. Así mismo, Charerntantanakul *et al.* (2006) detectaron AN en animales vacunados sólo después del desafío con una cepa virulenta. En consecuencia, ninguno de estos dos estudios pudo detectar AN tras la administración de una sola dosis de vacuna atenuada. Contrariamente, dos semanas después de vacunar con una sola dosis de vacuna atenuada de Aujeszky ya es posible detectar AN en el 100% de los animales (Meier *et al.,* 2003). El uso de un adyuvante convencional con una vacuna atenuada de PRRSV no mejora la respuesta humoral neutralizante, aunque sí que la mejora con una vacuna atenuada de Aujeszky (Meier *et al.,* 2003).

En resumen, tras la vacunación con vacunas atenuadas de PRRSV se observan unas evoluciones de las respuestas celulares y humorales neutralizantes bajas y lentas (Meier *et al.,* 2003; Royaee *et al.,* 2004). Además, parece que existe una elevada variabilidad individual de los animales en dichas respuestas (Meier *et al.,* 2003; Royaee *et al.,* 2004).

En algunos países, el uso de las vacunas atenuadas está restringido a los animales en crecimiento, ya que en las cerdas gestantes puede llegar a provocar serios problemas. Dewey *et al.* (2004) observaron que las cerdas gestantes vacunadas con vacunas atenuadas pueden presentar un descenso en el número de lechones nacidos vivos y dar lugar al nacimiento de lechones sanos infectados. Estos fenómenos se han asociado a la capacidad que tiene el virus de cruzar la placenta e infectar al feto en las fases más tardías de la gestación (Benfield *et al.,* 1997; Lager y Mengeling, 1995).

Las vacunas atenuadas presentan también otros inconvenientes. Los PRRSV atenuados pueden provocar estados virémicos y pueden excretarse; por tanto, tienen la capacidad de transmitirse a los cerdos no vacunados (Botner *et al.,* 1997). Además, el PRRSV vacunal atenuado puede revertir (Madsen *et al.,* 1998; Storgaard *et al.,* 1999; Nielsen *et al.,* 2001) y ser el causante de casos clínicos (Botner *et al.,* 1997; Mengeling *et al.,* 1999; Nielsen *et al.,* 2001 y 2002). En Dinamarca ha podido demostrarse que la administración de la vacuna atenuada del genotipo

americano fue la causante de múltiples casos de abortos y lechones nacidos débiles (Botner *et al.,* 1997; Madsen *et al.,* 1998). En EEUU, algunos aislados virulentos también se han relacionado con el uso de vacunas atenuadas (Opriessnig *et al.,* 2002).

Evaluando 20 cepas virulentas que tenían su origen en la cepa vacunal americana, Storgaard *et al.* (1999) pudieron demostrar que la reversión a cepa virulenta estaba asociada al cambio de sólo dos nucleótidos. Por tanto, dada la elevada tasa de mutación del virus, las probabilidades de reversión parecen ser muy elevadas.

En el caso de las vacunas atenuadas de origen español, el virus también puede cruzar la placenta y replicarse en el feto, si bien de modo más esporádico (Scortti *et al.,* en prensa). A pesar de ello, parece que ninguna de las dos vacunas analizadas tiene un efecto negativo en los parámetros reproductivos, sino todo lo contrario (Scortti *et al.,* en prensa). Según estos autores, la falta de transmisión del virus entre lechones durante la lactación indica que posiblemente estas cepas tienen una capacidad de propagarse inferior a las americanas.

1.8.2.2 Vacunas inactivadas

Comparadas con las vacunas atenuadas, la principal ventaja de las vacunas inactivadas es su incapacidad de diseminarse y de revertir a la virulencia, por lo que en algunos países son las únicas permitidas para la vacunación de las cerdas reproductoras. Tras la vacunación no es posible detectar el RNA del virus ni en sangre ni en muestras orofaríngeas (Nilubol *et al.,* 2004). Por el contrario, las vacunas inactivadas inducen una estimulación de la respuesta inmune más pobre que las atenuadas, si bien algunos autores han demostrado una eficacia parcial tanto en condiciones experimentales como en condiciones de campo (Swenson *et al.,* 1995; Plana-Duran *et al.,* 1997a; Reynaud *et al.,* 1998).

Respecto a la respuesta humoral inducida, las vacunas inactivadas no son capaces de estimular la creación de AN (Meier *et al.,* 2003). Tal y como ocurre con las vacunas atenuadas, la producción de AN no mejora con la administración de un adyuvante convencional. Curiosamente, el título de AN aumenta de forma significativa cuando los cerdos vacunados con vacunas inactivadas se infectan con una cepa virulenta de PRRS, pero no sucede así con la producción de anticuerpos no neutralizantes (Nilubol *et al.,* 2004). Por lo tanto, las vacunas no inducen ningún tipo de respuesta humoral neutralizante en cerdos no infectados, pero sí lo hacen en

cerdos ya infectados o infectados tras la vacunación (Baker *et al.,* 1999; Joo *et al.,* 1999; Nilubol *et al.,* 2004).

Respecto a la respuesta celular inducida por las vacunas inactivadas, Nilubol *et al.* (2004) no observaron un aumento significativo de la frecuencia de CP-IFN-γ específicas de virus ni siquiera después del desafío. Sin embargo, un estudio reciente ha demostrado que existe una producción de IFN-γ significativa y una estimulación de linfocitos $CD4^+/CD8^+$ y $CD8^+$ por parte de la vacuna inactivada (Piras *et al.,* 2005).

1.8.2.3 Mejora de las vacunas convencionales

Tal y como se ha explicado anteriormente, las vacunas convencionales estimulan una respuesta inmune que en ocasiones puede ser insuficiente. Además, la variabilidad genética puede llegar a ser un gran obstáculo en la obtención de una protección completamente efectiva. Con el objetivo de mejorar la eficacia de las vacunas frente al PRRSV se han planteado una serie de alternativas que se basan principalmente en 1) potenciar la respuesta celular Th1 y de AN y 2) favorecer la respuesta heteróloga hacia el mayor número de cepas posible.

Una de las estrategias más estudiadas para mejorar las vacunas, es la de administrar junto a la vacuna convencional una citoquina recombinante, un plásmido que codifique para dicha citoquina o cualquier otro sistema que favorezca la creación o liberación de estas citoquinas. Las citoquinas con las que más se ha trabajado son la IL-12 (Wee *et al.,* 2001; Foss *et al.,* 2002; Meier *et al.,* 2004; Charerntantanakul *et al.,* 2006) y el IFN-α (Meier *et al.,* 2004; Royaee *et al.,* 2004; Charerntantanakul *et al.,* 2006).

La IL-12 es un componente clave en el desarrollo de la inmunidad celular específica ya que estimula la conversión de los linfocitos T vírgenes en linfocitos Th1 y estimula la producción de IFN-γ por parte de los linfocitos T y las células NK (Kobayashi *et al.,* 1989; Chan *et al.,* 1991; Abbas *et al.,* 2000). La sola administración de la citoquina recombinante en cerdos infectados con el PRRSV, provoca un descenso del título del virus en pulmón y en sangre y previene de forma significativa el retraso en el crecimiento provocado por la infección (Carter y Curiel, 2005).

La eficacia de la administración de IL-12 junto a vacunas inactivadas del virus de Aujeszky está demostrada (Zuckermann *et al.,* 1998; Zuckermann *et al.,* 1999). En el caso del PRRSV, cuando

la IL-12 se ha administrado con ambos tipos de vacunas, atenuada o inactivada, se ha observado un aumento significativo de la respuesta celular específica (Wee *et al.,* 2001; Foss *et al.,* 2002; Meier *et al.,* 2004; Charerntantanakul *et al.,* 2006). Sin embargo, este aumento fue de carácter temporal y no han podido relacionarse tras la infección experimental ni con un descenso de la viremia ni con una mejora de la sintomatología (Foss *et al.,* 2002; Meier *et al.,* 2004; Charerntantanakul *et al.,* 2006). La administración de IL-12 no mejoró en ninguno de estos tres estudios la respuesta humoral neutralizante, a pesar de que todos observaron la aparición de anticuerpos no neutralizantes a las 2 semanas post-vacunación.

El IFN-α desempaña un papel muy importante frente a las infecciones víricas, pero como hemos visto antes el PRRSV puede inhibir la producción de esta citoquina (Albina *et al.,* 1998a). El uso del IFN-α para mejorar la respuesta inmune inducida por una vacuna atenuada, provoca un aumento temporal de la frecuencia de CP-IFN-γ específicas de PRRSV (Meier *et al.,* 2004; Royaee *et al.,* 2004). A pesar de ello, tras la infección no pudo relacionarse el aumento de la respuesta de IFN-γ con el descenso de la viremia (Meier *et al.,* 2004) ni con la reducción de la sintomatología (Charerntantanakul *et al.,* 2006). Tal y como ocurría con la IL-12, no se mejoró tampoco la producción de AN y los anticuerpos no neutralizantes aparecen en grandes cantidades a las 2 semanas post-vacunación (Meier *et al.,* 2004; Charerntantanakul *et al.,* 2006).

Otra de las vías estudiadas para mejorar la respuesta innata de las vacunas atenuadas es la administración de la toxina del cólera. Esta proteína es un potente inductor de la respuesta inflamatoria en el cerdo (Murtaugh y Foss, 2002) y de la presentación de antígeno (Foss *et al.,* 1999). Foss *et al.* (2002) observaron un aumento de la respuesta humoral específica hacia la ORF 5 cuando administraba la toxina del cólera con una vacuna atenuada de PRRSV; sin embargo, no pudieron observar un incremento significativo de la respuesta celular.

Con el objetivo de aumentar la capacidad de la respuesta hacia cepas heterólogas, una de las estrategias que se han propuesto es desarrollar vacunas que contengan más de una cepa (Mengeling *et al.,* 2003a y 2003b; Pesch *et al.,* 2005). Hasta el momento, uno de los pocos estudios existentes cuestiona en cierta medida la seguridad de este tipo de vacunas, ya que los cerdos inmunizados con una vacuna compuesta por 5 cepas presentaron un incremento en el tamaño de los linfonodos incluso antes del desafío (Mengeling *et al.,* 2003a). Mediante la administración simultánea de una vacuna atenuada y péptidos derivados de las ORFs 5 de varias cepas, Charerntantanakul *et al.* (2006) no pudieron observar una mejoría significativa en la

clínica con respecto al uso único de la vacuna atenuada. Esta combinación provocó un aumento en la respuesta de IFN-γ pero no estimuló la producción de AN.

Por otro lado, se ha intentado mejorar la respuesta con múltiples inmunizaciones con vacunas inactivadas o atenuadas o con combinaciones de estas. Los cerdos que reciben múltiples vacunaciones con cepas atenuadas o inactivadas, sufren un descenso en las poblaciones de linfocitos T efectores y de linfocitos T de memoria, así como en la producción de anticuerpos neutralizantes (Baker *et al.,* 1999; Bassaganya-Riera *et al.,* 2004). Es interesante destacar que en estos estudios, el uso de la combinación de múltiples inmunizaciones con vacunas inactivadas más una dosis final de vacuna atenuada o viceversa provocaba un descenso de la producción de AN, pero no de poblaciones T. En consecuencia, se presupone que la múltiple inmunización provoca una supresión de las respuestas humorales y, quizás en menos grado, de las respuestas celulares (Baker *et al.,* 1999; Bassaganya-Riera *et al.,* 2004).

1.8.3 Vacunas no convencionales

Debido a los problemas que presentan las vacunas convencionales, existe una amplia investigación en el uso de vacunas de DNA, con la administración o no de citoquinas como potenciadores de la respuesta.

Kwang *et al*. (1999) observaron que la administración de un plásmido que contenía la ORF 4 o la ORF 5 provocaba un aumento en la linfoproliferación o en la producción de IFN-γ, así como la mejora de los títulos de AN, pero no de anticuerpos no neutralizantes. A pesar de ello, la eficacia de las vacunas de DNA frente a la infección del PRRSV se considera muy limitada. Barfoed *et al.* (2004) evaluaron todas las ORFs, una por una y mezclas de ellas, mediante un desafío homólogo, observando viremia en todos los cerdos desde los 7 hasta los 32 días post-infección. En este mismo estudio, todas las ORFs provocaron la producción de AN tras la infección. Los mayores títulos de AN se observaron en todas aquellas combinaciones que incluían la ORF 5, mientras que las mayores cantidades de anticuerpos no neutralizantes estaban relacionadas con las combinaciones que incluían la ORF 7.

Para potenciar la respuesta de las vacunas de DNA también se ha estudiado la co-administración de citoquinas. La IL-2 es una potente activadora de la proliferación y de la funcionalidad de linfocitos T, B y células NK. Su uso como adyuvante de una vacuna de DNA de

ORF 5 o de ORF 7 protegió al pulmón de las lesiones provocadas por la infección del PRRSV y disminuyó el nivel de replicación del virus (Xue *et al.,* 2004). Sin embargo, el porcentaje de los animales en los que tuvo un efecto protector fue tan sólo del 33%. El mismo estudio observó un efecto protector sólo en el 66% de los cerdos vacunados con la ORF 5 y el IFN-γ.

Objetivos

Todos somos aficionados, en nuestra corta vida no tenemos tiempo para otra cosa

Charlie Chaplin

2. OBJETIVOS

1. Caracterizar de forma exhaustiva la respuesta inmune durante la infección de lechones con una cepa española del virus del síndrome reproductivo y respiratorio porcino.

2. Caracterizar la respuesta inmune desarrollada tras la vacunación de lechones con vacunas atenuadas del genotipo europeo del virus del síndrome reproductivo y respiratorio porcino y evaluar la protección que ofrecen frente a un desafío homólogo o heterólogo.

Publicaciones

Hay que tener cuidado con los libros de salud, podemos morir por culpa de una errata

Mark Twain

3. PUBLICACIONES

3.1 ESTUDIO 1

Immune responses of pigs after experimental infection with a European strain of Porcine reproductive and respiratory syndrome virus

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Immune responses of pigs after experimental infection with a European strain of *Porcine reproductive and respiratory syndrome virus*

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The purpose of this experiment was to study the immune response of pigs during an experimental infection with a European strain of *Porcine reproductive and respiratory syndrome virus* (PRRSV). Five pigs were challenged intranasally with PRRSV strain VP21 and another five were kept as controls. Clinical course and humoral and cell-mediated responses were monitored for 70 days post-infection (p.i.). Infected pigs developed mild signs at 24 h p.i. Viraemia was detectable by nested RT-PCR until day 14 p.i. Earliest seroconversions (ELISA) were seen by day 7 p.i. (three of five animals) and, by day 14, all inoculated pigs had seroconverted (ELISA and immunoperoxidase monolayer assay). Virus-neutralizing antibodies were undetectable until day 56 p.i. and, by day 70 p.i., two inoculated pigs still were negative. Flow-cytometry assays using peripheral blood mononuclear cells (PBMC) showed an upshift in $CD8^+$ cells (day 7 p.i.) and a downshift of $CD21^+$ cells (days 7 and 28 p.i.). Regarding cell-mediated responses, development of PRRSV-specific gamma interferon-secreting cells (IFN-γ-SC) and interleukin 4-secreting cells (IL4-SC) in PBMC was examined by ELISPOT assay. IFN-γ-SC were not detected significantly until day 14 p.i., whereas, for IL4-SC, no differences between groups were seen. Concurrently with the onset of viraemia and the development of clinical signs, serum haptoglobin levels and interleukin 10 (IL10) in PRRSV-stimulated PBMC-culture supernatants increased significantly. These differences disappeared later on. For IL2, IL4, IL8 or transforming growth factor beta, no differences were seen among groups. These results are compatible with a model in which the immune response does not fully control the outcome of the infection.

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INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) was first identified in 1991 by Dutch researchers (Wensvoort *et al.*, 1991) as the causative agent of a new disease of pigs. Since then, PRRSV has become one of the leading causes of economic losses in swine operations worldwide. At present, this virus is classified in the family *Arteriviridae* together with *Equine arteritis virus*, *Simian hemorrhagic fever virus* and *Lactate dehydrogenase-elevating virus* (Meulenberg *et al.*, 1994). Two genotypes are recognized (American and European). Both are thought to derive from a common ancestor, but genetic similarity between these two genotypes is about 55–65 % (Meng *et al.*, 1995; Murtaugh *et al.*, 1995; Gagnon & Dea, 1998; Dea *et al.*, 2000). In addition, genetic diversity of strains within a given genotype is high (Drew *et al.*, 1997; Forsberg *et al.*, 2002; Goldberg *et al.*, 2003; Mateu *et al.*, 2003).

Immune response to PRRSV is poorly understood but, in spite of this, some vaccines made from European- or American-type strains are being commercialized. Several studies have reported controversial results about the efficacy of vaccination (Meng, 2000). It seems that American-type vaccines are more effective to protect against infections caused by American-type strains than against European-type infections (van Woensel *et al.*, 1998; Lager *et al.*, 1999). However, some degree of protection against the heterologous genotype is observed (Lager *et al.*, 1999; Labarque *et al.*, 2003). This fact indicates that critical determinants of the immune response against each type of strains are similar, but not equal. As a consequence, at present there is no single vaccine that can claim full protection against all strains of PRRSV.

One of the reasons for the lack of development of newer and more efficacious vaccines against PRRSV is the scarce

knowledge on the immune response of pigs after infection by field strains. Available data have mostly been obtained by using American-type strains and have indicated that the adaptative immune response against American-type PRRSV is unique in its features. Infected piglets can be viraemic for up to 6–12 weeks, but circulating antibodies can be easily detected much earlier (Yoon *et al.*, 1995; Batista *et al.*, 2004; Johnson *et al.*, 2004). Nevertheless, neutralizing antibodies (NA) do not appear until 8–10 weeks post-infection (p.i.) and their role in protection is not clear (Murtaugh *et al.*, 2002). Besides, cell-mediated immune responses, measured as virus-specific gamma interferon-secreting cells (IFN-γ-SC), are erratic during the first weeks p.i. Later, there is a sudden increase in IFN-γ-SC that is maintained, despite a progressive fading of PRRSV-specific antibodies. However, the IFN-γ-SC response seems to be delayed, compared with other pathogens (Meier *et al.*, 2003).

To date, there has been no report showing the humoral and cell-mediated evolution of the immune response after infection with a wild PRRSV strain of the European genotype. The aim of the present study was to characterize the immune responses of pigs after infection with a European-type field strain of PRRSV.

METHODS

Animals and housing. Ten healthy 4-week-old Landrace pigs were selected in a high-health farm belonging to the Institut de Recerca i Tecnologia Agroalimentària (IRTA, Barcelona, Spain) that has historically been free of all major pig diseases, including PRRS. Animals were transported to the experimental facilities and raised until they were 16 weeks old, being examined periodically for antibodies against porcine circovirus type 2, Aujeszky's disease virus, *Porcine parvovirus*, swine influenza and *Mycoplasma hyopneumoniae*. All animals were seronegative for all of the above-mentioned pathogens. Also, animals were confirmed to be free of PRRSV, as determined by ELISA (HerdChek PRRS 2XR; IDEXX Laboratories).

Virus and challenge. The virulent PRRSV VP21 strain used for the challenge was isolated in porcine alveolar macrophages (PAM) from sera of naturally infected pigs during an outbreak of PRRS affecting a breeding farm located in the north of Spain in December 1991. That outbreak was characterized by abortions, stillbirths and, later on, piglet mortality. The virus was shown to be of the European genotype by sequencing of ORFs 2–7. Viral stock was done by passage ($n=3$) in PAM and titrated by means of the immunoperoxidase monolayer assay (IPMA). Before use, the viral stock was checked for bacterial contamination and for the presence of other viruses. Challenge was done when pigs were 17 weeks old. Pigs were divided randomly in two groups (A and B). Pigs in group A were inoculated intranasally with 2 ml viral suspension containing 10^6 $TCID_{50}$ PRRSV VP21 strain ml^{-1}, whilst pigs in group B (controls) received 2 ml sterile PBS.

Clinical examination and production parameters. Experimental pigs were clinically examined daily and rectal body temperatures were recorded from days 0 to 14 p.i. Every week, pigs were weighed and the amount of feedstuff consumed was recorded.

Samples. Blood samples were collected in duplicate (heparinized and siliconized blood-collecting tubes) immediately before challenge and then at days 7, 14, 21, 28, 56 and 70 p.i. Sera were used for PRRSV-specific RT-nested PCR (nPCR), determination of humoral responses and evaluation of haptoglobin and interleukin 8 (IL8), IL10 and transforming growth factor beta (TGF-β) levels. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood samples and used in flow-cytometry assays and for *in vitro* experiments.

Detection of viraemia. Serum samples were inoculated on MARC-145 and PAM cultures and incubated at 37 °C in 5 % CO_2 for 90 min. Then, sera were removed and minimal essential medium (Sigma) was added. Cultures were incubated for 3 days at 37 °C in 5 % CO_2. Infection of inoculated cells was determined by IPMA.

Besides viral isolation, sera were examined by nPCR. Briefly, total RNA was extracted from 150 µl serum by using a commercial system (Nucleospin RNA virus; Macherey-Nagel). Total RNA was transcribed and cDNA was used in a first PCR round directed to viral ORF5 (Mateu *et al.*, 2003). This first-round PCR had a sensitivity of about 10^2 $TCID_{50}$ $(ml\ serum)^{-1}$. PCR products were used in an nPCR (forward primer, 5′-TCTTGCTTCTGGTGGCTTTT-3′; reverse primer, 5′-CATGTTT-GATGGTGACGAGG-3′) that produced a 499 bp amplicon. Cycling parameters for both PCRs were: 94 °C for 45 s; 55 °C for 45 s; 72 °C for 45 s for a total of 35 cycles. Under these conditions, the nPCR was considered to be able to detect <10 viral copies $(ml\ serum)^{-1}$.

Humoral immune response. A commercially available ELISA (HerdChek PRRS 2XR; Idexx Laboratories) was used to measure PRRSV-specific antibodies. According to the manufacturer, sample to positive control (S/P) ratios of $>0{\cdot}4$ were considered positive. PRRSV antibody titres were also determined in sera by using IPMA as described by Wensvoort *et al.* (1991), using MARC-145 cells infected with the PRRSV VP21 strain. Tests were done in duplicate.

NA were measured by the technique described by Yoon *et al.* (1994) and Jusa *et al.* (1996). Tests were done in triplicate by using whole serum, inactivated serum and serum plus 10 % fresh guinea-pig complement. Briefly, 50 µl of each serum to be tested was diluted serially from 1/20 to 1/160 in cell-culture medium. Dilutions were mixed with 50 µl viral suspension containing 200 $TCID_{50}$ of the PRRSV strain VP21 (with or without complement). Virus–serum mixtures were incubated for 1 h at 37 °C and then added to MARC-145 cultures in duplicate (96-well plates) and incubated for 3 days at 37 °C in 5 % CO_2. Infection of cell cultures was revealed by using IPMA.

Total serum antibodies (IgG and IgM) were analysed by means of a commercial capture ELISA (Pig IgG ELISA quantification kit and Pig IgM ELISA quantification kit; Bethyl Laboratories), done according to the recommendations of the manufacturer. Serum-antibody concentrations were calculated by using a regression line calculated after measurement of ODs of the standards provided with the kit.

Isolation and culture of PBMC. PBMC were separated from whole blood by density-gradient centrifugation with Histopaque 1077 (Sigma). For PBMC cultures, RPMI 1640 medium supplemented with 10 % fetal calf serum (Invitrogen), 1 mM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 5 mM 2-mercaptoethanol (Sigma), 50 000 IU penicillin l^{-1} (Invitrogen), 50 mg streptomycin l^{-1} (Invitrogen) and 50 mg gentamicin l^{-1} (Sigma) was used. Trypan blue was used to assess viability.

Flow-cytometry assays. Phenotypic analysis of PBMC subsets was done by flow cytometry using the following mAbs: anti-CD4–PE (phycoerythrin) (clone 74-12-4, provided by Dr J. Domínguez, INIA, Madrid, Spain), anti-CD8–FITC (fluorescein isothiocyanate) (clone 76-2-11, INIA), anti-CD21–FITC (4530-02; Southern Biotechnologies), anti-CD25 (K231-3B2, INIA), anti-SWC3–PE (4525-09, Southern Biotechnologies) and anti-SLA-II DR (clone 1F12, INIA).

When needed, goat $F(ab')_2$ anti-mouse IgG_{2a} R-PE-conjugated

antibody and goat F(ab′)$_2$ anti-mouse IgG$_1$ FITC-conjugated antibody (Southern Biotechnologies) were used as secondary antibodies. Irrelevant, isotype-matched antibodies were included as background controls. Analyses were done by using an EPICS XL-MLC cytometer (Coulter) to an excitation wavelength of 488 nm and with 580 and 630 nm filters.

ELISPOT: PRRSV-specific IFN-γ-SC and IL4-secreting cells (IL4-SC). Frequencies of PRRSV-specific IFN-γ-SC and IL4-SC in PBMC were analysed by an ELISPOT assay using commercial mAbs (Swine IFN-γ and Swine IL4 Cytosets; Biosource Europe) according to a previously reported method (Díaz & Mateu, 2005). Briefly, for IFN-γ, ELISA plates (Costar 3590; Corning) were coated overnight with 8·3 μg IFN-γ capture antibody ml^{-1} diluted in carbonate/bicarbonate buffer (pH 9·6). Plates were then washed and blocked for 1 h at 37 °C with 150 μl PBS with 1 % BSA. After removal of the blocking solution, 5×10^5 PBMC were dispensed per well (50 μl) and stimulated with the VP21 strain at an m.o.i. of 0·1 as the recall antigen. After 20 h incubation at 37 °C in a 5 % CO_2 atmosphere, cells were removed and the biotinylated detection antibody was added at 2·5 μg ml^{-1} (50 μl) and incubated for 1 h at 37 °C. The reaction was revealed by sequential incubation of plates with streptavidin–peroxidase (1 h) and insoluble TMB blue (Calbiochem). For IL4, the protocol was similar, but working dilutions of the mAbs were 9·6 μg ml^{-1} (capture antibody diluted in PBS, pH 7·2) and 5·0 μg ml^{-1} (biotinylated detection antibody). In both cases, unstimulated cells and phytohaemagglutinin (PHA)-stimulated controls (10 μg ml^{-1}) were also included.

To calculate the PRRSV-specific frequencies of IFN-γ-SC and IL4-SC, counts of spots in unstimulated wells were subtracted from counts in virus-stimulated wells. Frequencies of cytokine-producing cells were expressed as responding cells in 10^6 PBMC.

Cytokine (IL2, IL4, IL8, IL10 and TGF-β) and haptoglobin ELISAs. PBMC were seeded at a density of 2×10^6 cells per well (250 μl) in 96-well plates and were mock-stimulated or stimulated with either PRRSV strain VP21 (m.o.i. of 0·1) or PHA (10 μg ml^{-1}). After 24 h incubation at 37 °C in 5 % CO_2, cell-culture supernatants were collected and frozen at −80 °C until needed. Capture ELISAs for IL2, IL4 and IL10 were performed as reported previously (Darwich *et al.*, 2003; Díaz & Mateu, 2005), using commercial pairs of mAbs (Swine IL2, IL4 and IL10 cytosets; Biosource Europe). For IL8, an ELISA kit was used (IL8 Immunoassay kit; Biosource Europe). For TGF-β, an ELISA was developed by using commercial antibodies (TGF-β cytosets; Biosource Europe). The cut-off point of each ELISA was calculated as the mean + 3SD OD of negative controls. Cytokine concentrations were calculated by using the linear-regression formula from ODs of the cytokine standards provided by the manufacturer. IL8, IL10 and TGF-β were also measured in sera of experimental animals by using the above-mentioned ELISAs. Serum-haptoglobin levels were determined by means of ELISA (Haptoglobin assay phase range; Tridelta) according to the manufacturer's instructions.

Statistical analysis. The regression line of cytokine standards in ELISA was calculated by using SPSS v. 12.0 (SPSS Inc.). Statistical comparisons between groups (Mann–Whitney test) were done by using StatsDirect v. 2.4.1. All tests done in the study, as well as the statistical analysis, were performed blind.

RESULTS

Clinical course and viraemia

In infected animals, the first signs of disease were seen at 24 h p.i. Pigs were dull, anorectic and had a fever (>39·5 °C). During the first week, all infected pigs had a fever but, by day 7 p.i., rectal temperatures had returned to normality. Between days 7 and 11 p.i., a second peak of fever was seen in three of five challenged animals (Fig. 1). Significant differences in weight gain were observed during the first 2 weeks of infection. From days 0 to 7, challenged pigs gained 5·1 kg less than uninfected controls and, from days 7 to 14, 2·6 kg less ($P<0·05$). On the third week, challenged pigs showed a compensatory growth and, by day 35 p.i., weight gains were equal in infected and uninfected pigs (Table 1).

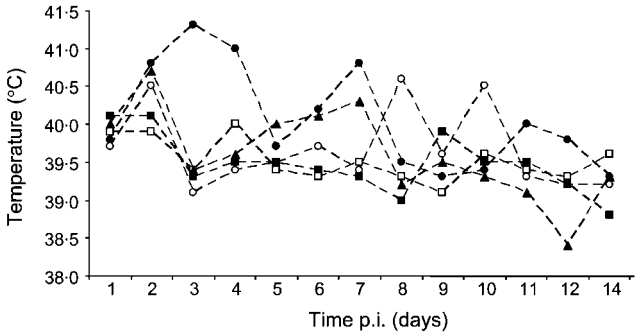

Fig. 1. Rectal body temperatures were measured in all pigs (five infected and five controls) at 24 h intervals during the first 2 weeks p.i. The graph only shows the results of PRRSV-infected pigs, as control pigs always had normal body temperatures. Each symbol (●, ○, ▲, ■ and □) corresponds to one of the five pigs of the challenge group.

All challenged animals were positive by nPCR at least once during the first 2 weeks p.i. However, viraemia was probably low as, with a single-round RT-PCR, only one animal was detected as positive and the remaining viraemic pigs were detected only after nPCR. Viral isolation was less sensitive than nPCR, only being isolated from four pigs. None of the uninoculated pigs yielded nPCR-positive results or viral isolation.

Table 1. Weekly weight gain of PRRSV-infected pigs and uninfected controls

From 35 days p.i. to the end of the experiment, both groups showed the same weight gain.

Time p.i. (days)	Mean ± SD weekly weight gain (kg)	
	Uninfected	Infected
0–7	11·1 ± 1·08*	6 ± 4·60
7–14	7·9 ± 1·38*	5·3 ± 2·56
14–21	8·1 ± 0·74	7·1 ± 1·08
21–28	4·8 ± 0·75	7·5 ± 1·27
28–35	1·1 ± 0·74	1·1 ± 0·75

*$P<0·05$.

Humoral immune response

All pigs were seronegative to PRRSV at day 0. By using ELISA, two infected pigs seroconverted by day 7 (mean S/P ratio, 0·51) and all were clearly positive by day 14 (mean S/P ratio, 2·12). ELISA titres remained high throughout the 70 days of observation and a slow decline was seen from day 56 p.i. onwards (mean S/P ratio at day 70 p.i., 1·81). IPMA was slightly less sensitive than ELISA. With IPMA, positive results were only obtained from day 14 p.i. onwards. The highest mean IPMA titres were seen at day 56 p.i. [$\log_{10}$(titre) = 3·05].

NA were first detected at day 56 p.i. and only in three of five infected pigs. The remaining two pigs had not developed NA by day 70. By using whole serum, the highest neutralizing titre was $\log_{10}$(titre) = 1·80. Addition of fresh complement increased titres by one dilution. All uninoculated controls remained seronegative to PRRSV in all tests throughout the study. Table 2 summarizes these results.

Total antibodies were determined by using specific pig IgG and IgM ELISAs. Although great individual variability was seen in both groups for both IgG and IgM, comparison of mean values between groups did not show significant differences.

Flow-cytometry assays

At day 7 p.i., infected pigs had a significant increase in the proportion of peripheral blood $CD8^+$ cells and a decrease in $CD21^+$ cells ($P<0·05$). At day 14 p.i., these differences disappeared. Infected pigs again had a decrease in $CD21^+$ cells at 28 days p.i. No differences were seen among groups regarding expression of CD4, CD25, SWC3 or SLA-II in PBMC. Table 3 shows the complete results of the flow-cytometry analysis.

ELISPOT

In contrast to anti-PRRSV-antibody values, the frequencies of PRRSV-specific IFN-γ-SC had some variability in all samples of the infected group. Notwithstanding, a similar evolution of PRRSV-specific IFN-γ-SC was observed in all infected animals. PRRSV-specific IFN-γ-SC were detected for the first time at day 14 p.i. (49 IFN-γ-SC in 10^6 PBMC, $P<0·05$). Then, counts of PRRSV-specific IFN-γ SC showed highs and lows until day 70 p.i., when they reached a peak (62 IFN-γ-SC in 10^6 PBMC) (Fig. 2). Regarding IL4-SC, values were low (<10 in 10^6 PBMC) and no significant differences were seen among groups.

Cytokine ELISAs

Examination of PRRSV-stimulated PBMC-culture supernatants showed no significant differences among groups for IL2, IL4 or TGF-β (data not shown). In contrast, at 1 week p.i., levels of IL10 were significantly higher ($P<0·05$) in culture supernatants of PRRSV-stimulated PBMC (>80 pg ml^{-1}) obtained from infected pigs. This difference was not observed later in the course of infection, although PBMC from challenged pigs always produced more IL10 against PRRSV than did cells from control animals (Fig. 2). IL8, IL10 and TGF-β secretion was also examined by ELISA in sera. None of the sera yielded positive results for these cytokines.

In PRRSV-challenged pigs, serum-haptoglobin levels were increased significantly at days 7 and 14 p.i. ($P<0·05$), returning to values similar to those of uninfected pigs by day 21 p.i. (Fig. 3).

DISCUSSION

The immune response of pigs against PRRSV is not yet fully understood and some phenomena, such as the delayed development of NA and the role of T cell-mediated

Table 2. Serological evolution of PRRSV-infected pigs by ELISA (HerdChek PRRS 2XR; Idexx Laboratories), IPMA and viral-neutralization test (VNT)

Results are expressed as the mean ± SD titre. Uninfected animals were negative in all assays.

Test	Development of specific PRRSV-antibodies (days p.i.)						
	0	7	14	21	28	56	70
ELISA							
No. positive	−	2/5	5/5	5/5	5/5	5/5	5/5
S/P ratio		0·51 ± 0·14	2·12 ± 0·20	2·23 ± 0·24	2·17 ± 0·26	1·76 ± 0·3014	1·81 ± 0·25
IPMA							
No. positive	−	−	5/5	5/5	5/5	5/5	5/5
Mean $\log_{10}$(titre)			2·57 ± 0·33	2·75 ± 0·13	2·99 ± 0·16	3·05 ± 0·13	2·69 ± 0·27
VNT							
No. positive	−	−	−	−	−	3/5	3/5
Mean $\log_{10}$(titre)						1·80 ± 0·28	1·60 ± 0·42

Table 3. Flow-cytometry results for PBMC of PRRSV-infected pigs and uninfected controls

Results are expressed as mean ± SD (%) for five PRRSV-infected and five control pigs.

Marker	Proportion of PBMC ± SD (%) (days p.i.)				
	0	7	14	21	28
CD4					
Uninfected	18·9 ± 0·5	19·8 ± 2·8	13·3 ± 4·6	14·8 ± 1·9	17·2 ± 1·8
Infected	20·8 ± 3·4	18·9 ± 2·5	18·0 ± 4·3	16·2 ± 3·2	14·3 ± 5·7
CD8					
Uninfected	31·3 ± 7·7	24·0 ± 4·7	23·9 ± 5·1	25·3 ± 6·5	31·9 ± 3·4
Infected	23·0 ± 3·0	34·6 ± 6·6*	25·4 ± 2·0	27·2 ± 3·8	27·4 ± 3·6
CD21					
Uninfected	10·1 ± 2·2	8·4 ± 2·7	6·9 ± 2·7	5·5 ± 2·6	9·0 ± 1·4
Infected	14·7 ± 5·1	3·9 ± 1·9*	5·4 ± 1·8	5·7 ± 1·7	7·4 ± 0·9*
CD25					
Uninfected	5·6 ± 1·3	8·2 ± 1·8	7·3 ± 1·2	9·4 ± 4·6	10·6 ± 1·2
Infected	6·5 ± 1·2	6·7 ± 0·6	6·9 ± 0·9	9·9 ± 2·0	10·6 ± 1·2
SWC3					
Uninfected	6·1 ± 2·7	9·1 ± 2·8	7·1 ± 1·7	14·2 ± 1·9	8·5 ± 1·6
Infected	9·5 ± 3·0	9·1 ± 4·0	7·8 ± 5·4	13·1 ± 1·3	12·2 ± 6·6
SLA-II					
Uninfected	50·7 ± 7·1	50·4 ± 9·5	47·3 ± 9·5	53·3 ± 10·9	53·8 ± 12·5
Infected	51·9 ± 3·9	55·1 ± 3·9	48·8 ± 5·1	54·3 ± 8·6	55·7 ± 6·8

*$P < 0{\cdot}05$.

immunity, remain poorly explained. Most immunological studies have been done by using American-type strains (Osorio *et al.*, 2002; Meier *et al.*, 2003; Royaee *et al.*, 2004) and, as genetic diversity is thought to play a role in the immune escape of the virus, differences might arise between the immune responses in European- and American-type PRRSV infections. Some studies analysed the role of immunity after the infection or vaccination of pigs with a European-genotype PRRSV (Nielsen & Bøtner, 1997; López Fuertes *el al.*, 1999; Samsom *et al.*, 2000; Aasted *et al.*, 2002; Suradhat & Thanawongnuwech, 2003), but a global immunological picture is still lacking.

Our experiment was deliberately conducted with relatively aged pigs (17 weeks of age). This selection was done firstly because we wished to have a clear model of infection in which maturation of the immune system was not a source of confusion and, secondly, because we tried to avoid

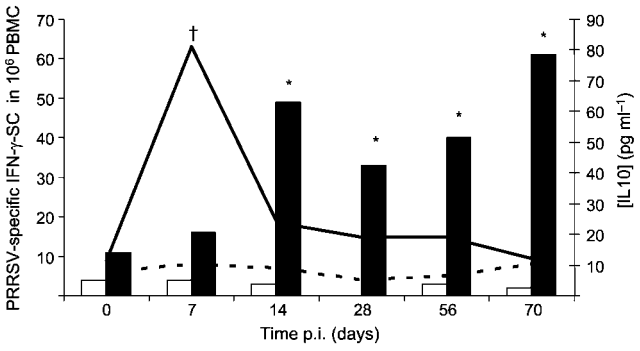

Fig. 2. IFN-γ-SC (ELISPOT) and IL10 (ELISA) levels in PBMC-culture supernatants after recall of PRRSV in pigs infected with the VP21 strain. For IL10, results are expressed as the mean of secreted IL10 (pg ml^{-1}), whereas specific virus IFN-γ-SC are expressed as the mean frequency of the specific virus IFN-γ-SC in 10^6 PBMC. Empty bars, IFN-γ-SC in control pigs; filled bars, IFN-γ-SC in infected pigs; dashed line, IL10 in control pigs; solid line, IL10 in infected pigs. *, $P < 0{\cdot}05$; †, $P < 0{\cdot}05$.

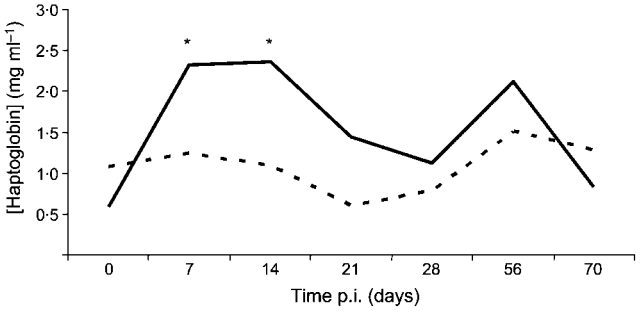

Fig. 3. Kinetics of serum haptoglobin as measured by ELISA after infection with a PRRSV European-type strain. Dashed line, control pigs; solid line, infected pigs. *, $P < 0{\cdot}05$.

development of a severe disease that could kill animals before the planned end of the experiment.

After experimental infection with the PRRSV VP21 strain, all infected pigs developed only mild signs of disease in the first 24 h p.i. These signs, mainly fever, anorexia and weight loss, lasted until 14 days p.i., when viraemia ceased. This course of infection is similar to that described in other reports using European-type strains (van der Linden *et al.*, 2003).

Interestingly, viraemia was low and was only detectable after nPCR was used. These facts suggest that clinical signs are related to the presence of virus in blood. As PRRSV does not replicate in monocytes or other blood cells (Duan *et al.*, 1997), it would be expected that viraemia reflected the intensity of virus replication in tissues. Other experimental PRRSV infections or vaccinations have also shown short viraemias, even in pigs younger than ours (López Fuertes *et al.*, 1999; Meier *et al.*, 2003; Sipos *et al.*, 2003).

By using ELISA, development of specific antibodies could be seen as early as 7 days p.i. and lasted until the end of the study at detectable levels. The performance of IPMA was somewhat poorer but, by day 14 p.i., all infected animals were detected as seropositive. It is known that detection of early antibodies is useful as a diagnostic tool, but it seems that they do not play a role in protection against the infection (reviewed by Murtaugh *et al.*, 2002; Lopez & Osorio, 2004).

In our experiment and as reported by others (Vezina *et al.*, 1996; Meier *et al.*, 2003), NA appeared late (from day 56 p.i. onwards) and not in all animals. The existence of decoy immunodominant epitopes in GP5 has been suggested as the cause of such delayed production (Ostrowski *et al.*, 2002).

The role of NA in protection against PRRSV is controversial. Osorio *et al.* (2002) showed that NA could be protective against PRRSV if administered exogenously at high doses. However, most studies showed that the production of NA after an experimental infection or vaccination is low and sporadic, with considerable individual variability (Loemba *et al.*, 1996; Meier *et al.*, 2003). In our case, clearance of viraemia was observed before NA could be detected. We cannot rule out the possibility that very small amounts of NA, undetectable in neutralization assays, could have existed at that time and played a role in the cessation of viraemia but, as three different methods of examining serum NA were used, it seems more reasonable to think that viraemia disappeared in the absence of NA. In immunodeficient mice infected with *Lactate dehydrogenase-elevating virus*, a related arterivirus, viraemia can also disappear without noticeable NA (Onyekaba *et al.*, 1989).

One of the effects attributed to PRRSV is the induction of a polyclonal B activation (Lamontagne *et al.*, 2001). In our case, neither total IgM nor IgG levels rose significantly after infection. Also, the proportion of neither $CD21^+$ nor $CD25^+$ cells increased. Moreover, the proportion of $CD21^+$ cells in blood decreased at 1 week p.i. Mobilization of B cells to tissues could explain these results and would be consistent with the suggestions of other authors, which have indicated that, in PRRS, polyclonal activation of B cells occurs in lymphoid organs, but not in blood (Kawashima *et al.*, 1999; Lamontagne *et al.*, 2001).

In contrast, $CD8^+$ cells increased in blood in the first week p.i. Samsom *et al.* (2000) observed that $CD8^+$ cells also increased in bronchoalveolar fluid of pigs after a PRRSV infection. However, this increase is not likely to be attributable to a clonal expansion of PRRSV-specific T cells. If this was the case, other evidence, such as IL2 production, IFN-γ increase or an upshift of $CD25^+$ cells, should have been observed. Other reports (Shimizu *et al.*, 1996; Albina *et al.*, 1998) indicated that this was not a mitogenic effect of the virus, but a response to some physiological stimulus. As we will discuss later, this increase in $CD8^+$ cells correlated with the detection of IL10 in PBMC-culture supernatants and with the raise of haptoglobin levels in serum. Moore *et al.* (2001) showed that IL10 can promote growth and differentiation of $CD8^+$ cells.

Regarding the cellular immune response, our results showed that PRRSV-specific IFN-γ-SC appeared at week 2 p.i. Levels of IFN-γ-SC were undulant in the first 70 days p.i. This evolution agrees with the results of Meier *et al.* (2003) although, in our case, the strength of the response, in terms of the number of IFN-γ-SC, was lower. Whether these differences were caused by the use of different mAbs in ELISPOT or reflect a true difference cannot be known at this time.

Interestingly, although the initial raise of PRRSV-specific IFN-γ-SC was delayed for 14 days after the challenge, the last detection of viraemia in infected pigs corresponded to the appearance of these cells. This fact may be incidental, but suggests some role of IFN-γ-SC in the control of the infection. Regarding IL4-SC, no significant responses against PRRSV were detected in infected pigs. This is surprising, considering the fast and high humoral response against the virus, but might be an explanation for the delayed development of NA.

In our study, results for IL2, IL4 and IL8 did not show significant differences between control and infected animals. These observations reinforce the notion that some type of immunomodulation takes place in the course of infection (Molitor *et al.*, 1997; Lager & Mengeling, 2000). We also evaluated IL10 responses of infected pigs. Other authors showed that this cytokine can play a significant role in PRRS (Chung & Chae, 2003; Suradhat & Thanawongnuwech, 2003; Suradhat *et al.*, 2003). In our case, the ability of PBMC to produce IL10 in response to viral stimulation was a significant fact in the first week p.i., but IL10 was not detected significantly in cell-culture supernatants afterwards. This pattern is difficult to explain.

It is known that IL10 is produced mainly by cells of the monocyte/macrophage lineage, by regulatory T cells or, less frequently, by B cells. At this time, none of these populations can be ruled out as the source of this cytokine. Besides, TGF-β levels were not significantly different in serum or cell-culture supernatants of infected and uninfected pigs and, as we discussed before, IL4 was not detected in response to PRRSV. Natural regulatory T cells are thought to belong to the $CD4^+CD25^+$ subset (McGuirk & Mills, 2002). We did not see any change in the relative proportion of these two markers in PBMC throughout the study and, under this perspective, it seems unlikely that natural regulatory T cells were involved in IL10 production. It is tempting to think of monocytes as being responsible for this production. However, this needs further research to be clarified.

The increased ability of PBMC to produce IL10 against PRRSV at the first week p.i. correlated with the rise of serum-haptoglobin levels. Haptoglobin is an acute-phase protein produced by the liver and related to IL6 (Asai *et al.*, 1999). It seems to play a major role in modulating immune responses through a complex network of interactions. Thus, haptoglobin release has been related to the secretion of anti-inflammatory cytokines, particularly IL10, through the interaction with CD163, a haemoglobin-scavenger receptor that is solely present in cells of the monocyte/macrophage lineage (Moestrup & Moller, 2004; Philippidis *et al.*, 2004). Interestingly, other pig viruses, such as *African swine fever virus*, replicate mainly in $CD163^+$ macrophages (Sánchez-Torres *et al.*, 2003).

In summary, comparison of our results with previous studies performed with American strains (Meier *et al.*, 2003; Royaee *et al.*, 2004) suggests that the immune response in all PRRSV infections is similar. Main traits are a strong, non-neutralizing humoral response with a delayed development of NA, a low IFN-γ-SC frequency, very low or undetectable IL2 and IL4 responses and, in the first stages of infection, a significant involvement of IL10 and haptoglobin.

As suggested by Xiao *et al.* (2004), the results presented in this work are consistent with a hypothetical model in which the outcome of PRRSV infection is related more to the dynamics of permissive macrophages and the early events of the natural response than to the development of specific immunity. In such a model, the earliest events in the course of the infection would involve release of IL10 and haptoglobin. This IL10, either released by infected macrophages as an anti-inflammatory response or induced by viral proteins, would probably impair, but not abolish, the development of cell-mediated immunity (Royaee *et al.*, 2004). This fact would be reflected in the erratic levels of virus-specific IFN-γ-SC during the first weeks p.i. (Meier *et al.*, 2003) and in the lack of virus-specific IL2- and IL4-producing cells. As infection progresses, the cytolytic cycles of virus replication would decrease the number of permissive macrophages (Xiao *et al.*, 2004). As seen by others (Duan *et al.*, 1997), permissive cells should account for only a small proportion of total tissue macrophages. The stronger the cytolytic cycle, the faster the resolution of the viraemia. At this point, the weak cell-mediated immunity would be able to confine PRRSV to certain tissues (i.e. lymph nodes) where the proportion of permissive cells could be still relatively high (Xiao *et al.*, 2004). In consequence, viraemia should cease or become inconstant and of low level. This would occur regardless of whether or not NA had developed. However, as long as the virus persisted in tissues, local IL10 release (or other cytokines) would keep T-cell responses low. As far as the infection can be confined to certain tissues and permissive macrophages are not replaced at a high rate, the number of infected cells will decline steadily and the immune response will finally be able to clear the infection. This moment would correspond with the rise of blood IFN-γ-SC and the final development of NA. From then onwards, the pig would be protected against homologous challenge (Mengeling *et al.*, 2003). Such a model would explain why no clear correlation between the immune response and the clearing of infection can be determined, as well as making sense of the delayed T-cell responses observed in PRRSV infection. In our opinion, this hypothesis deserves further study, as it can give a whole image of the events taking place in PRRSV infection and can contribute to understanding how to develop new and more efficient vaccines.

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REFERENCES

Aasted, B., Bach, P., Nielsen, J. & Lind, P. (2002). Cytokine profiles in peripheral blood mononuclear cells and lymph node cells from piglets infected in utero with porcine reproductive and respiratory syndrome virus. *Clin Diagn Lab Immunol* **9**, 1229–1234.

Albina, E., Piriou, L., Hutet, E., Cariolet, R. & L'Hospitalier, R. (1998). Immune responses in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Immunol Immunopathol* **61**, 49–66.

Asai, T., Mori, M., Okada, M., Uruno, K., Yazawa, S. & Shibata, I. (1999). Elevated serum haptoglobin in pigs infected with porcine reproductive and respiratory syndrome virus. *Vet Immunol Immunopathol* **70**, 143–148.

Batista, L., Pijoan, C., Dee, S., Olin, M., Molitor, T., Joo, H. S., Xiao, Z. & Murtaugh, M. (2004). Virological and immunological responses to porcine reproductive and respiratory syndrome virus in a large population of gilts. *Can J Vet Res* **68**, 267–273.

Chung, H.-K. & Chae, C. (2003). Expression of interleukin-10 and interleukin-12 in piglets experimentally infected with porcine reproductive and respiratory syndrome virus (PRRSV). *J Comp Pathol* **129**, 205–212.

Darwich, L., Balasch, M., Plana-Durán, J., Segalés, J., Domingo, M. & Mateu, E. (2003). Cytokine profiles of peripheral blood mononuclear cells from pigs with postweaning multisystemic wasting syndrome in response to mitogen, superantigen or recall viral antigens. *J Gen Virol* **84**, 3453–3457.

Dea, S., Gagnon, C. A., Mardassi, H., Pirzadeh, B. & Rogan, D. (2000). Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. *Arch Virol* **145**, 659–688.

Díaz, I. & Mateu, E. (2005). Use of ELISPOT and ELISA to evaluate IFN-γ, IL-10 and IL-4 responses in conventional pigs. *Vet Immunol Immunopathol* (in press).

Drew, T. W., Lowings, J. P. & Yapp, F. (1997). Variation in open reading frames 3, 4 and 7 among porcine reproductive and respiratory syndrome virus isolates in the UK. *Vet Microbiol* **55**, 209–221.

Duan, X., Nauwynck, H. J. & Pensaert, M. B. (1997). Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Microbiol* **56**, 9–19.

Forsberg, R., Storgaard, T., Nielsen, H. S., Oleksiewicz, M. B., Cordioli, P., Sala, G., Hein, J. & Bøtner, A. (2002). The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. *Virology* **299**, 38–47.

Gagnon, C. A. & Dea, S. (1998). Differentiation between porcine reproductive and respiratory syndrome virus isolates by restriction fragment length polymorphism of their ORFs 6 and 7 genes. *Can J Vet Res* **62**, 110–116.

Goldberg, T. L., Lowe, J. F., Milburn, S. M. & Firkins, L. D. (2003). Quasispecies variation of porcine reproductive and respiratory syndrome virus during natural infection. *Virology* **317**, 197–207.

Johnson, W., Roof, M., Vaughn, E., Christopher-Hennings, J., Johnson, C. R. & Murtaugh, M. P. (2004). Pathogenic and humoral immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) are related to viral load in acute infection. *Vet Immunol Immunopathol* **102**, 233–247.

Jusa, E. R., Inaba, Y., Kouno, M., Hirose, O., Shibata, I., Kubota, M. & Yasuhara, H. (1996). Slow-reacting and complement-requiring neutralizing antibody in swine infected with porcine reproductive and respiratory syndrome (PRRS) virus. *J Vet Med Sci* **58**, 749–753.

Kawashima, K., Narita, M. & Yamada, S. (1999). Changes in macrophage and lymphocyte subpopulations of lymphoid tissues from pigs infected with the porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Immunol Immunopathol* **71**, 257–262.

Labarque, G., Van Gucht, S., Van Reeth, K., Nauwynck, H. & Pensaert, M. (2003). Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines. *Vet Microbiol* **95**, 187–197.

Lager, K. M. & Mengeling, W. L. (2000). PRRS: nature of the RNA virus and how it causes disease (a keynote paper). In *Proceedings of the 16th International Pig Veterinary Society Congress*, pp. 538–543. 17–20 September 2000, Melbourne, Australia.

Lager, K. M., Mengeling, W. L. & Brockmeier, S. L. (1999). Evaluation of protective immunity in gilts inoculated with the NADC-8 isolate of porcine reproductive and respiratory syndrome virus (PRRSV) and challenge-exposed with an antigenically distinct PRRSV isolate. *Am J Vet Res* **60**, 1022–1027.

Lamontagne, L., Page, C., Larochelle, R., Longtin, D. & Magar, R. (2001). Polyclonal activation of B cells occurs in lymphoid organs from porcine reproductive and respiratory syndrome virus (PRRSV)-infected pigs. *Vet Immunol Immunopathol* **82**, 165–182.

Loemba, H. D., Mounir, S., Mardassi, H., Archambault, D. & Dea, S. (1996). Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. *Arch Virol* **141**, 751–761.

Lopez, O. J. & Osorio, F. A. (2004). Role of neutralizing antibodies in PRRSV protective immunity. *Vet Immunol Immunopathol* **102**, 155–163.

López Fuertes, L., Doménech, N., Alvarez, B., Ezquerra, A., Domínguez, J., Castro, J. M. & Alonso, F. (1999). Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. *Virus Res* **64**, 33–42.

Mateu, E., Martín, M. & Vidal, D. (2003). Genetic diversity and phylogenetic analysis of glycoprotein 5 of European-type porcine reproductive and respiratory virus strains in Spain. *J Gen Virol* **84**, 529–534.

McGuirk, P. & Mills, K. H. G. (2002). Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. *Trends Immunol* **23**, 450–455.

Meier, W. A., Galeota, J., Osorio, F. A., Husmann, R. J., Schnitzlein, W. M. & Zuckermann, F. A. (2003). Gradual development of the interferon-γ response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. *Virology* **309**, 18–31.

Meng, X. J. (2000). Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. *Vet Microbiol* **74**, 309–329.

Meng, X. J., Paul, P. S., Halbur, P. G. & Lum, M. A. (1995). Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. *Arch Virol* **140**, 745–755.

Mengeling, W. L., Lager, K. M., Vorwald, A. C. & Koehler, K. J. (2003). Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **93**, 13–24.

Meulenberg, J. J., Hulst, M. M., de Meijer, E. J., Moonen, P. L., den Besten, A., de Kluyver, E. P., Wensvoort, G. & Moormann, R. J. (1994). Lelystad virus belongs to a new virus family, comprising lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus. *Arch Virol Suppl* **9**, 441–448.

Moestrup, S. K. & Moller, H. J. (2004). CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. *Ann Med* **36**, 347–354.

Molitor, T. W., Bautista, E. M. & Choi, C. S. (1997). Immunity to PRRSV: double-edged sword. *Vet Microbiol* **55**, 265–276.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* **19**, 683–765.

Murtaugh, M. P., Elam, M. R. & Kakach, L. T. (1995). Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. *Arch Virol* **140**, 1451–1460.

Murtaugh, M. P., Xiao, Z. & Zuckermann, F. (2002). Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. *Viral Immunol* **15**, 533–547.

Nielsen, J. & Bøtner, A. (1997). Hematological and immunological parameters of 4½-month old pigs infected with PRRS virus. *Vet Microbiol* **55**, 289–294.

Onyekaba, C. O., Harty, J. T., Even, C., Hu, B.-G. & Plagemann, P. G. W. (1989). Persistent infection of mice by lactate dehydrogenase-elevating virus: effects of immunosuppression on virus replication and antiviral immune responses. *Virus Res* **14**, 297–315.

Osorio, F. A., Galeota, J. A., Nelson, E., Brodersen, B., Doster, A., Wills, R., Zuckermann, F. & Laegreid, W. W. (2002). Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. *Virology* **302**, 9–20.

Ostrowski, M., Galeota, J. A., Jar, A. M., Platt, K. B., Osorio, F. A. & Lopez, O. J. (2002). Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. *J Virol* **76**, 4241–4250.

Philippidis, P., Mason, J. C., Evans, B. J., Nadra, I., Taylor, K. M., Haskard, D. O. & Landis, R. C. (2004). Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxygenase-1 synthesis: antiinflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. *Circ Res* **94**, 119–126.

Royaee, A. R., Husmann, R. J., Dawson, H. D., Calzada-Nova, G., Schnitzlein, W. M., Zuckermann, F. A. & Lunney, J. K. (2004). Deciphering the involvement of innate immune factors in the development of the host response to PRRSV vaccination. *Vet Immunol Immunopathol* **102**, 199–216.

Samsom, J. N., de Bruin, T. G. M., Voermans, J. J. M., Meulenberg, J. J. M., Pol, J. M. A. & Bianchi, A. T. J. (2000). Changes of leukocyte phenotype and function in the broncho-alveolar lavage fluid of pigs infected with porcine reproductive and respiratory syndrome virus: a role for $CD8^+$ cells. *J Gen Virol* **81**, 497–505.

Sánchez-Torres, C., Gómez-Puertas, P., Gómez-del-Moral, M., Alonso, F., Escribano, J. M., Ezquerra, A. & Domínguez, J. (2003). Expression of porcine CD163 on monocytes/macrophages correlates with permissiveness to African swine fever infection. *Arch Virol* **148**, 2307–2323.

Shimizu, M., Yamada, S., Kawashima, K., Ohashi, S., Shimizu, S. & Ogawa, T. (1996). Changes of lymphocyte subpopulations in pigs infected with porcine reproductive and respiratory syndrome (PRRS) virus. *Vet Immunol Immunopathol* **50**, 19–27.

Sipos, W., Duvigneau, C., Pietschmann, P. & 7 other authors (2003). Parameters of humoral and cellular immunity following vaccination of pigs with a European modified-live strain of porcine reproductive and respiratory syndrome virus (PRRSV). *Viral Immunol* **16**, 335–346.

Suradhat, S. & Thanawongnuwech, R. (2003). Upregulation of interleukin-10 gene expression in the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus. *J Gen Virol* **84**, 2755–2760.

Suradhat, S., Thanawongnuwech, R. & Poovorawan, Y. (2003). Upregulation of IL-10 gene expression in porcine peripheral blood mononuclear cells by porcine reproductive and respiratory syndrome virus. *J Gen Virol* **84**, 453–459.

van der Linden, I. F. A., Voermans, J. J. M., van der Linde-Bril, E. M., Bianchi, A. T. J. & Steverink, P. J. G. M. (2003). Virological kinetics and immunological responses to a porcine reproductive and respiratory syndrome virus infection of pigs at different ages. *Vaccine* **21**, 1952–1957.

van Woensel, P. A., Liefkens, K. & Demaret, S. (1998). European serotype PRRSV vaccine protects against European serotype challenge whereas an American serotype vaccine does not. *Adv Exp Med Biol* **440**, 713–718.

Vezina, S. A., Loemba, H., Fournier, M., Dea, S. & Archambault, D. (1996). Antibody production and blastogenic response in pigs experimentally infected with porcine reproductive and respiratory syndrome virus. *Can J Vet Res* **60**, 94–99.

Wensvoort, G., Terpstra, C., Pol, J. M. & 19 other authors (1991). Mystery swine disease in the Netherlands: the isolation of Lelystad virus. *Vet Q* **13**, 121–130.

Xiao, Z., Batista, L., Dee, S., Halbur, P. & Murtaugh, M. P. (2004). The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. *J Virol* **78**, 5923–5933.

Yoon, I. J., Joo, H. S., Goyal, S. M. & Molitor, T. W. (1994). A modified serum neutralization test for the detection of antibody to porcine reproductive and respiratory syndrome virus in swine sera. *J Vet Diagn Invest* **6**, 289–292.

Yoon, K. J., Zimmerman, J. J., Swenson, S. L. & 7 other authors (1995). Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. *J Vet Diagn Invest* **7**, 305–312.

3.2 ESTUDIO 2

Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs

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Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs

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Abstract

Immunization of piglets with two different European-type modified live vaccines against porcine reproductive and respiratory syndrome (PRRS) virus produced different outcomes. After vaccination, pigs became viremic (42 days), neutralizing antibodies did not develop, and frequencies of virus-specific gamma-interferon-secreting cells (IFN-γ-SC) were low. Levels of interleukin-10 (IL-10) produced by peripheral blood mononuclear cells (PBMC) seemed to inversely correlate with interferon-gamma responses. After a challenge with a virulent Spanish strain, one vaccine (V3) protected piglets against viremia while the other (V1) did not. The vaccine V3 induced the highest IFN-γ-SC frequencies. IL-2, IL-4 or transforming growth factor-beta responses were not detected at any time for neither of the vaccines. In contrast, haptoglobin rose in sera of viremic pigs after the challenge. These results indicated a strong involvement of IFN-γ, and maybe IL-10, in the development of immunity against PRRS virus.

Keywords: Porcine reproductive and respiratory virus; Interferon-gamma; Interleukin-10; vaccine

Introduction

Porcine reproductive and respiratory syndrome (PRRS) emerged in Europe in the early years of the 1990 decade and rapidly spread all over the continent becoming one of the major causes of economic losses for swine producers. Simultaneously, the disease emerged in USA with a similar impact on pig production. This syndrome, caused by an arterivirus (Meulenberg et al., 1994), is characterized in its reproductive form by abortions, stillbirths and weakly born piglets and, in young pigs, causes pneumonia and weight gain losses. PRRS virus (PRRSV) is a small positive-stranded polyadenylated RNA virus that contains nine open reading frames (ORFs). ORFs 1a and 1b comprise 80% of the genome and encode the RNA polymerase; ORFs 2 to 5 encode four structural glycoproteins (GP2, GP3, GP4 and GP5) and a small non-glycosylated protein called 2b (Wu et al., 2005); ORF6 encodes a non-glycosylated integral membrane protein (M) and ORF7 encodes the nucleocapside protein (N) (Conzelmann et al., 1993; Meulenberg et al., 1997; Snijder and Meulenberg, 1998). Currently, it is known that two distinct genotypes of the virus exist, the European and the American, that share only 55–80% similarity (Meng et al., 1995).

Soon after the discovery of the virus vaccines were marketed. The first ones were a modified live virus (MLV) vaccine called RespPRRS made of an American-type strain (Christopher-Hennings et al., 1997; Dee and Joo, 1997; Mengeling et al., 1996), and an inactivated vaccine called Cyblue, made of a European strain (Plana-Duran et al., 1997). From then, several vaccines, both attenuated and inactivated, have been commercialized.

Notwithstanding, none of the vaccines can claim full protection against the disease. On one hand, PRRSV, either wild type or attenuated, induces a low level of cell-mediated immunity (Meier et al., 2003) and neutralizing antibodies (NA)

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Table 1
Percentages of similarity between the PRRSV strains used in the study

	LV	V1	V3	VP21
ORF 2				
LV	–	94.6	98.0	94.6
V1	94.6	–	94.3	98.3
V3	98.0	94.3	–	93.9
VP21	94.6	98.3	93.9	–
ORF 3				
LV	–	94.2	98.3	94.0
V1	94.2	–	92.5	99.4
V3	94.2	92.5	–	93.3
VP21	94.4	99.6	93.9	–
ORF 4				
LV	–	94.2	99.0	94.5
V1	94.2	–	94.2	99.2
V3	99.0	94.2	–	93.5
VP21	94.5	99.2	94.5	–
ORF 5				
LV	–	94.9	98.9	94.5
V1	94.9	–	94.4	99.6
V3	98.9	94.4	–	94.0
VP21	94.5	99.6	94.0	–
ORF 6				
LV	–	97.7	97.7	96.9
V1	97.7	–	100	98.6
V3	97.7	100	–	98.6
VP21	96.9	98.6	98.6	–
ORF 7				
LV	–	96.6	100	96.3
V1	96.6	–	96.6	99.7
V3	100	96.6	–	96.3
VP21	96.3	99.7	96.3	–

Lelystad virus (LV, Genbank accession number *M96262*) was used as a reference.
LV = Lelystad virus, V1 = Modified live vaccine 1, V3 = Modified live vaccine 2, VP21 = wild-type Spanish PRRSV strain used for the challenge.

do not develop until a late phase of the infection (Meier et al., 2003; Vezina et al., 1996; Yoon et al., 1995). In addition, the cytokine imbalance after a vaccination or an infection suggests a possible downregulation of many cytokines (Royaee et al., 2004; Van Reeth et al., 1999). On the other hand, genetic diversity of PRRSV is thought to influence the efficacy of vaccines under field conditions (Labarque et al., 2004; Pesch et al., 2005) although some degree of cross protection exists (Mengeling et al., 2003a, 2003b). With this panorama, the development of improved vaccines needs a deeper understanding of the immunity against PRRSV.

Up to date, only one report has dealt with the evolution of the immune responses, both humoral and cellular, after vaccination with a European type PRRS-modified live vaccine (MLV) (Sipos et al., 2003). However, that study was done with only one vaccine, did not challenge the animals and did not evaluate frequencies of IFN-γ-secreting cells (IFN-γ-SC), a parameter that seems to be related to protection (Meier et al., 2003). The aim of the present study was to characterize the humoral and the cellular responses of pigs in the course of a vaccination with two European-type PRRS-MLV vaccines and after a challenge with a European PRRS wild-type virus.

Results

Virus sequencing and phylogenetic analysis

Similarity between vaccines and VP21 strain was above 90%. Highest values were obtained for ORF7 and ORF6 and lowest similarities corresponded to envelope glycoproteins (Table 1). As a whole, VP21 was closer to V1 (both are Spanish strains) than to V3. Interestingly, in ORF6 both vaccines had exactly the same sequence that differed from VP21 and LV by a change in the codon starting at position 14,402 in LV genome (cga → caa). That change would correspond in the amino acid sequence to a substitution of an arginine by a glutamine.

Detection of PRRSV

Viral isolation attempts in PAM or MARC-145 cells from blood samples taken after vaccination were negative. In contrast, all vaccinated animals were positive by nPCR at least once during the first 6 weeks PV (Table 2). However, viremia was probably low as, with a single round RT-PCR, only six samples were positive during the first 14 days PV. After the challenge, only G1 pigs were detected as positive by nPCR (4/5) at 7 days post-infection (PI). Sequencing of the nPCR amplicons showed the virus to be the VP21 strain. None of the control pigs yielded nPCR-positive results or viral isolation.

Hematology

In all three groups, hematological values remained between normal ranges in the examined period (28 days PV). However, some parameters significantly changed in vaccinated pigs at first week PV, particularly for neutrophils, monocytes and platelets ($P < 0.05$). Table 3 summarizes these results.

Humoral immune response

All pigs were seronegative to PRRSV at day 0. By using ELISA, all vaccinated animals seroconverted by day 14 PV (mean *S*/*P* ratio, 2.5 ± 0.2 for G1 and 2.4 ± 0.6 for G2) (Table 4). ELISA titers peaked at 21 and 28 days PV for G1 and G2, respectively (G1 = 3.2 ± 0.3 and G2 = 3.1 ± 0.3). After the

Table 2
Evolution of viremia in vaccinated pigs as determined by RT-nested PCR

Group	Days post-vaccination								
	0	7	14	21	28	42	63 [a]	70	91
G1	0	5	4	5	2	1	0	4	0
G2	0	5	3	3	1	2	0	0	0

[a] At 63 days post-vaccination, both groups were challenged with 10^6 $TCID_{50}$ of a wild-type Spanish PRRSV strain (VP21).

Table 3
Evolution of hematological parameters of vaccinated pigs from day 0 to day 28 post-vaccination

Parameter/ Group	Days post-vaccination			
	0	7	14	28
Total leukocytes				
G1	16.1 ± 1.8	12.6 ± 2.9[a]	15.2 ± 2.0	13.4 ± 1.9
G2	16.1 ± 2.5	15.4 ± 1.0[b]	15.7 ± 4.4	12.5 ± 1.9
C	19.1 ± 4.2	12.8 ± 1.2[a]	12.2 ± 3.1	13.3 ± 2.2
%Neutrophils				
G1	49.7 ± 6.4	36.7 ± 9.2[a]	16.6 ± 3.4	25.0 ± 5.1
G2	43.7 ± 7.1	28.4 ± 4.1[b]	21.8 ± 4.6	21.2 ± 7.0
C	53.8 ± 8.1	39.8 ± 3.9[a]	24.7 ± 6.9	20.0 ± 5.0
Neutrophils (10^3/μl)				
G1	7.9 ± 1.3	4.6 ± 1.3[a]	2.4 ± 0.7	3.3 ± 0.6
G2	6.9 ± 1.2	3.3 ± 0.6[a]	3.3 ± 0.8	2.7 ± 1.1
C	10.3 ± 3.3	6.1 ± 0.9[c]	3.0 ± 1.0	2.7 ± 0.9
%Monocytes				
G1	3.8 ± 1.2	3.2 ± 0.5[a]	6.0 ± 2.2	4.9 ± 1.4
G2	3.1 ± 1.5	6.6 ± 2.2[b]	4.9 ± 2.2	5.2 ± 1.8
C	3.2 ± 2.0	5.1 ± 0.4[b]	4.6 ± 1.4	5.1 ± 2.5
Monocytes (10^3/μl)				
G1	0.6 ± 0.2	0.4 ± 0.1[a]	0.9 ± 0.4	0.7 ± 0.3
G2	0.5 ± 0.4	0.8 ± 0.3[b]	0.8 ± 0.7	0.7 ± 0.2
C	0.7 ± 0.5	0.8 ± 0.1[b]	0.5 ± 0.1	0.6 ± 0.5
Platelets (10^3/μl)				
G1	431 ± 111	347 ± 72[a]	515 ± 110	383 ± 96
G2	434 ± 120	489 ± 43[b]	573 ± 114	465 ± 91
C	564 ± 95	422 ± 37[b]	470 ± 88	518 ± 101

Superindexes indicate statistical differences (Kruskal–Wallis test using the Connover–Iman method for multiple comparisons; $P < 0.05$). Groups with the same superscripted letter were statistically equal (a, b, or c). The remaining cells subsets did not show statistical differences.

challenge with VP21, titers did not show any significant change. With IPMA, positive results were only obtained from day 14 PV onwards. The highest mean IPMA titers were seen at day 28 PI, namely 91 days PV ($\log_{10}$ = 3.2 ± 0.1 for G1 and 3.0 ± 0.3 for G2) (Table 4).

NA were only detected after the challenge (28 days PI) and only in four vaccinated pigs (two animals/group) (Table 4). By using whole serum, the highest titer was $\log_{10}$ (titer) = 1.3 ± 0.0 (G2). Addition of fresh complement increased titers by one dilution. All unvaccinated controls remained seronegative to PRRSV in all tests throughout the study.

IFN-γ-SC responses

Frequencies of IFN-γ-SC strongly depended on the strain used for in vitro stimulation. When V1 was used, IFN-γ-SC frequencies were very low in both vaccinated groups and did not exceed 15 IFN-γ-SC/10^6 PBMC (Fig. 1). In contrast, use of V3 for in vitro stimulation produced more clear results, with mean frequencies of IFN-γ-SC that ranged from as low as 12/10^6 PBMC (G1 at 28 days PV) to as high as 125/10^6 PBMC (G2 at day 28 PI, namely 91 days PV) (Fig. 2).

In vitro stimulation with VP21 strain produced results similar to V3 but of a slightly lower intensity (Fig. 3). Taking the results obtained with the VP21 stimulus as a common reference to all groups, PRRSV-specific IFN-γ-SC were firstly detected at 7 days PV (16/10^6 PBMC for G1 and 42/10^6 PBMC for G2) rising to >60/10^6 PBMC at 14 days PV ($P < 0.05$ compared to controls). Then frequencies of PRRSV-specific IFN-γ-SC decreased in both vaccinated groups (<40/10^6 PBMC). At 63 days PV, G1 showed a mean value of 23 IFN-γ-SC/10^6 PBMC and G2 values increased up to 80 IFN-γ-SC/10^6 PBMC ($P < 0.05$). After challenge (63 days PV), a booster effect was observed in both vaccinated groups, but the increase was more evident in G1. By day 28 PI, mean values of PRRS-specific IFN-γ-SC were about 115/10^6 PBMC in both groups.

Stimulation with V1, V3 or VP21 of PBMC from control unvaccinated and uninfected pigs yielded negative results in ELISPOT with no significant background.

Cytokine and haptoglobin ELISAs

In vitro stimulation of PBMC with V1, V3 or VP21 showed no significant differences among groups for IL-2 or IL-4 in cell culture supernatants (data not shown). Regarding IL-10, when V1 was used as stimulus, PBMC of control pigs produced high levels (110–322 pg/ml) (Fig. 4). In contrast, V3 and VP21 did

Table 4
Serological evolution of PRRS-MLV vaccinated pigs by ELISA (HerdCheck, Idexx Laboratories), immunoperoxidase monolayer assay (IPMA) and viral neutralization test (VNT)

Test	Vaccine	Days post-vaccination								
		0	7	14	21	28	42	63[a]	70	91
ELISA no. of positive S/P ratio	G1	Neg	Neg	5/5 2.5 ± 0.2	5/5 3.2 ± 0.3	5/5 3.1 ± 0.3	5/5 3.1 ± 0.3	5/5 2.8 ± 0.4	5/5 3.0 ± 0.1	5/5 3.0 ± 0.1
	G2	Neg	Neg	5/5 2.3 ± 0.6	5/5 2.9 ± 0.5	5/5 3.1 ± 0.3	5/5 3.1 ± 0.2	5/5 3.0 ± 0.4	5/5 2.9 ± 0.2	5/5 2.8 ± 0.1
IPMA no. of positive ($\log_{10}$ titer)	G1	Neg	Neg	5/5 2.3 ± 0.3	5/5 2.4 ± 0.2	5/5 2.8 ± 0.1	5/5 3.0 ± 0.3	5/5 2.6 ± 1.9	5/5 2.8 ± 0.4	5/5 3.2 ± 0.0
	G2	Neg	Neg	5/5 2.4 ± 0.2	5/5 2.4 ± 0.2	5/5 2.8 ± 0.2	5/5 2.9 ± 0.1	5/5 2.7 ± 0.3	5/5 2.8 ± 0.4	5/5 3.0 ± 0.3
VNT no. of positive ($\log_{10}$ titer)	G1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	2/5 1.2 ± 0.1
	G2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	2/5 1.3 ± 0.0

Results are expressed as the mean ± standard deviation titer.
Unvaccinated animals were negative in all assays.
[a] At 63 days post-vaccination, both groups were challenged with 10^6 $TCID_{50}$ of a wild-type Spanish PRRSV strain (VP21).

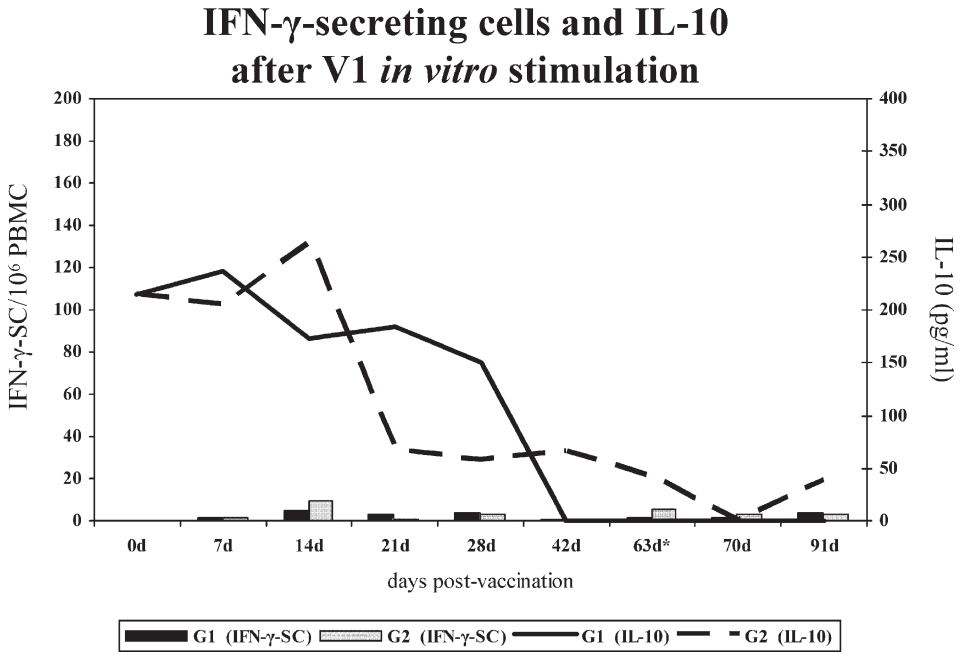

Fig. 1. The figure shows the frequency of IFN-γ-secreting cells (ELISPOT) and IL-10 levels in peripheral blood mononuclear cell culture supernatants (ELISA) after V1 in vitro stimulation in both vaccinated groups. For IL-10 results are expressed as the average of IL-10 secreted (pg/ml) (detection limit = 32 pg/ml), whereas specific-virus IFN-γ-secreting cells are expressed as the average frequency of the specific-virus IFN-γ-secreting cells by 10^6 PBMC. *At 63 days post-vaccination, both groups were challenged with 10^6 $TCID_{50}$ of a wild-type Spanish PRRSV strain (VP21).

not induce IL-10 secretion in cells of control pigs (<42 pg/ml; $P < 0.05$).

When the specific IL-10 response was examined in vaccinated pigs, stimulation of PBMC produced different results depending on the virus used. In G1 pigs, stimulation with V1 produced high levels of IL-10 until 42 days PV (150–236 pg/ml); from this date on, IL-10 could not be detected (Fig. 1). For G2 pigs, in vitro stimulation with V1 produced lower levels of IL-10 that began to decline by day 21 PV and became undetectable by day 70 PV. Stimulation of PBMC

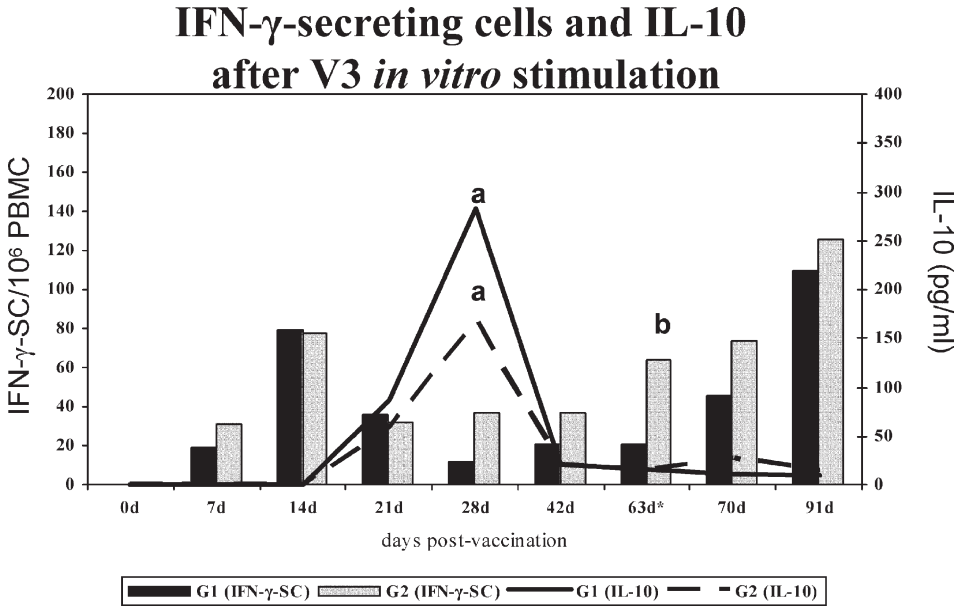

Fig. 2. The figure shows the frequency of IFN-γ-secreting cells (ELISPOT) and IL-10 levels in peripheral blood mononuclear cell culture supernatants (ELISA) after V3 in vitro stimulation in both vaccinated groups. For IL-10 results are expressed as the average of IL-10 secreted (pg/ml) (detection limit = 32 pg/ml), whereas specific-virus IFN-γ-secreting cells are expressed as the average frequency of the specific-virus IFN-γ-secreting cells by 10^6 PBMC. ([a]) G1 and G2 > Controls IL-10 levels ($P < 0.05$). ([b]) G2 > G1 IFN-γ-secreting cells ($P < 0.05$). *At 63 days post-vaccination, both groups were challenged with 10^6 $TCID_{50}$ of a wild-type Spanish PRRSV strain (VP21).

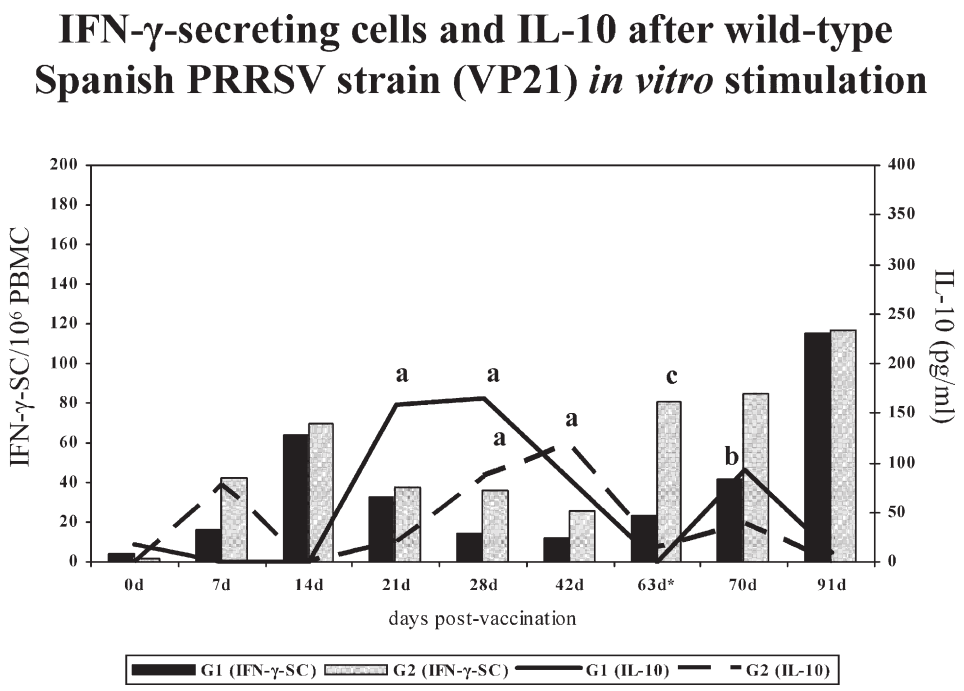

Fig. 3. The figure shows the frequency of IFN-γ-secreting cells (ELISPOT) and IL-10 levels in peripheral blood mononuclear cell culture supernatants (ELISA) after wild-type Spanish PRRSV strain (VP21) in vitro stimulation in both vaccinated groups. For IL-10 results are expressed as the average of IL-10 secreted (pg/ml) (detection limit = 32 pg/ml), whereas specific-virus IFN-γ-secreting cells are expressed as the average frequency of the specific-virus IFN-γ-secreting cells by 10^6 PBMC. ([a]) G1 and G2 > Controls IL-10 levels ($P < 0.05$). ([b]) G1 > G2 IL-10 levels ($P < 0.05$). ([c]) G2 > G1 IFN-γ-secreting cells ($P < 0.05$). *At 63 days post-vaccination, both groups were challenged with 10^6 $TCID_{50}$ of a wild-type Spanish PRRSV strain (VP21).

with V3 produced low IL-10 values in all animals, except at 28 days PV, when these levels significantly increased in both vaccinated groups (Fig. 2). Finally, use of VP21 as an in vitro stimulus yielded two IL-10 peaks in G1 pigs (between 21 and 28 days PV and 7 days PI) and one peak in G2 pigs (between 28 to 42 days PV) ($P < 0.05$, compared to control group). Interestingly, this phase of higher ability of PBMC for producing IL-10 after in vitro stimulation with the VP21 strain (between 21 and 42 days PV) corresponded to the lower IFN-γ-SC values (Fig. 3).

None of the sera yielded positive results for IL-10 and TGF-β. In contrast, serum haptoglobin levels in G1 increased

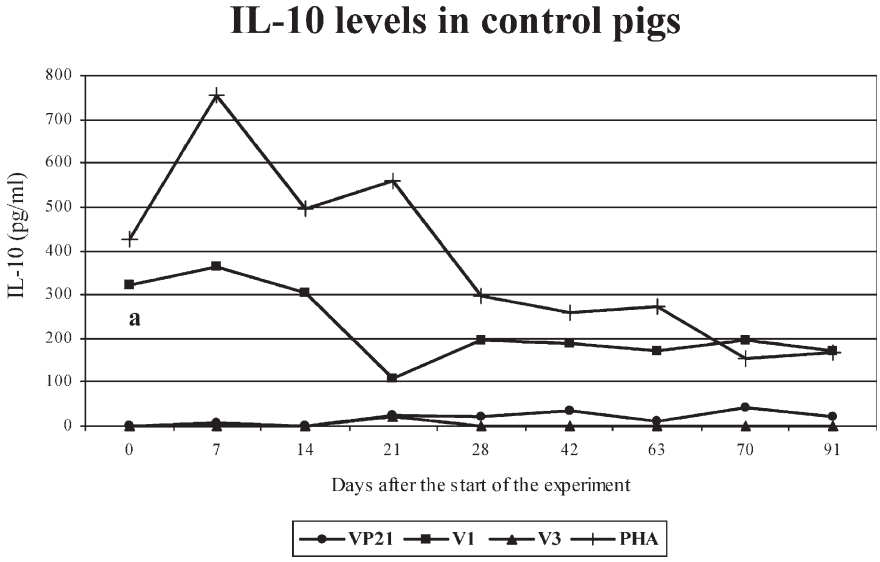

Fig. 4. IL-10 levels in peripheral blood mononuclear cells culture supernatants (ELISA) after wild-type Spanish PRRSV strain (VP21), PRRS-MLV made of a Spanish strain (V1), PRRS-MLV made of a European strain (V3) and PHA stimulation in vitro assays of unvaccinated and uninfected pigs (controls). Results are expressed as the average of IL-10 secreted (pg/ml) (detection limit = 32 pg/ml). ([a]) IL-10 levels after V1 in vitro stimulation always were significantly higher than V3 and VP21 ($P < 0.05$).

significantly at 7 days PI ($P < 0.05$), returning to normality by day 14 PI (Fig. 5).

Discussion

Several studies have reported that the immune response against arteriviruses is unique in its features (Balasuriya and MacLachlan, 2004; Cafruny et al., 1999; Meier et al., 2003). Regarding PRRSV, it seems that both, humoral and cellular, responses do not control the infection adequately. NA develop slowly and usually appear after the resolution of viremia. In addition, cell-mediated immunity also rises slowly, and virus-specific IFN-γ-SC frequencies are low compared to other viral pig diseases (Meier et al., 2003). Some authors suggested that, in PRRS, immunity might play a secondary role and stated that availability of permissive macrophages may determine the outcome of the infection (Diaz et al., 2005; Xiao et al., 2004). A similar hypothesis has been postulated for lactate dehydrogenase-elevating virus (LDV), a related arterivirus (Onyekaba et al., 1989; Van den Broek et al., 1997).

Vaccines made of MLV or inactivated PRRSV are being commercialized and have shown a relative efficacy (Mengeling et al., 2003a, 2003b; Plana-Duran et al., 1997; Van Woensel et al., 1998). Nevertheless, this is a controversial issue. Firstly, because it is unclear how well vaccines prevent the infection and how safe they are (Labarque et al., 2003; Mengeling et al., 2003a, 2003b) and, secondly, because it is thought that the genetic diversity of PRRSV can negatively affect the field efficacy of vaccines (Labarque et al., 2004; Meng, 2000).

In the present study, two European-type vaccines were examined. To assess differences in protection, it was important to evaluate the genetic similarity between them and the challenge VP21 strain. As expected, main differences between the PRRSV strains used were found in ORF3 and ORF5, which are known to be the most variable regions in the PRRSV genome (Pirzadeh et al., 1998; Forsberg et al., 2001).

Besides this, it is interesting to note that ORF6 of both vaccine viruses shared a change corresponding to the codon starting at position 14,402 in LV. This change (cga → caa) produced a substitution of an arginine by a glutamine compared to VP21 or LV. Grebennikova et al. (2004) showed that in the American-type PRRSV strain NADC-8, a change in the position 14,440 (alanine → threonine) contributed significantly to reduce viral virulence. In our case and theirs, one amino acid present in virulent strains was substituted by a polar uncharged one in the attenuated viruses. This finding supports the idea that this site in ORF6 can be related to attenuation.

After vaccination, both G1 and G2 pigs became viremic, and some animals remained so as late as 42 days PV. Sipos et al. (2003) reported 3 weeks of viremia in piglets using one of the European-type PRRSV MLV used in the present study. These extended periods of viremia could significantly contribute to the spread of vaccine virus in the pig population. This fact was reported for American-type vaccines used in Europe (Mortensen et al., 2002; Storgaard et al., 1999). These results point out the question of attenuation. Extended periods of viral replication should not be interpreted as equivalent to virulence or vice versa. As Xiao et al. (2004) suggested, in PRRS, viral replication would occur as long as permissive macrophages exist. Thus, with an attenuated PRRSV strain, that can be thought to produce less intense cytolytic cycles, viral replication should last a long time.

In our case, pigs vaccinated with V1 developed viremia when infected with VP21, while in G2 pigs virus was not detectable in blood after the challenge. Genetic similarity was higher between V1 and VP21 (98.3–99.7%) than between V3 and VP21 (92.3–98.6%). That fact shows that, in our case, the heterologous strain provided better protection than the homologous one. This apparent contradiction could be explained by

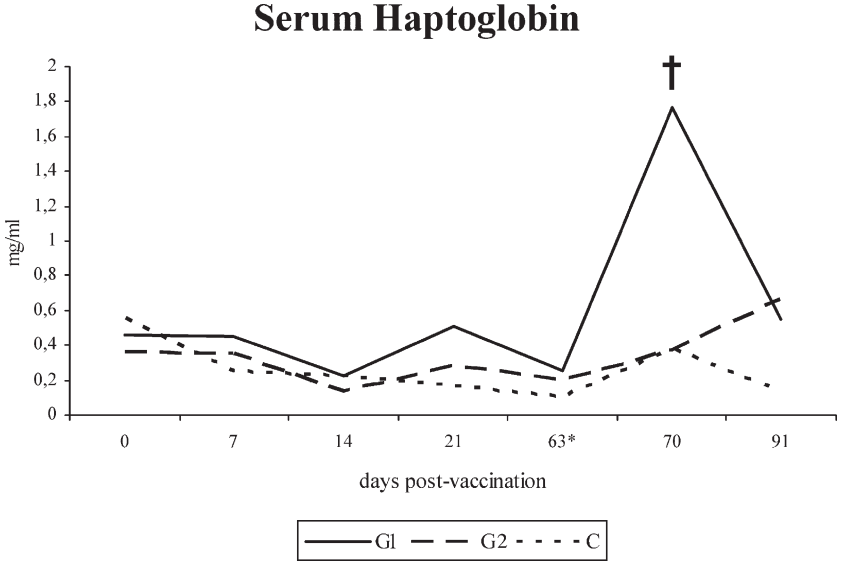

Fig. 5. Kinetics of serum haptoglobin as measured by ELISA in vaccinated and control pigs. (†)$P < 0.05$. *At 63 days post-vaccination both groups were challenged with a wild-type Spanish PRRSV strain (VP21).

the different adjuvants used with the vaccines or by differences in the ability of each vaccine strain to induce PRRSV-specific IFN-γ-SC. In addition, the capability of V1 to stimulate IL-10 responses might also have had a role.

Regarding the hematological parameters examined, we observed some changes at 7 days PV, but all values remained between the physiological ranges for pigs. This has also been reported by Sipos et al. (2003).

By using ELISA, development of specific antibodies could be seen at 14 days PV in all vaccinated pigs and titers remained high throughout the experiment. Interestingly, ELISA titers did not increase significantly after inoculation of the wild-type strain even in viremic pigs. Some authors claimed (Johnson et al., 2004) that the quantitation of the humoral response (total antibodies) measured by ELISA can serve as an indicator of the virulence of a given PRRSV strain. In the present case, using the same ELISA than Johnson et al. (2004), titers after vaccination reached a high of 3.15 (*S*/*P* ratio), similar to the values obtained with the most virulent strain used in Johnson's work. In our opinion, ELISA titers can not be used to measure virulence, at least, when European-type PRRSV strains are evaluated.

In our study, vaccinated pigs did not develop NA before the challenge. Meier et al. (2003), using a MLV vaccine made of an American-type strain, needed two doses of the vaccine to induce only in some animals low levels of NA. Although it seems that high titers of NA could play a role in the outcome of the infection (Lopez and Osorio, 2004) most experiments showed a low, delayed and sporadic development of NA (Meier et al., 2003; Mengeling et al., 1999; Vezina et al., 1996). However, as it has been observed in other arterivirus such as LDV, viremia can be cleared in absence of NA (Balasuriya and MacLachlan, 2004). In our experiment, G2 pigs achieved sterilizing immunity without the need of detectable NA.

Regarding the cellular immune response using VP21 or V3 as stimuli, levels of IFN-γ-SC rose at 14 days PV, then were erratic, and finally rose again at 63 days PV. This figure is similar to the data reported by us in a previous experiment, where unvaccinated pigs were experimentally infected with the VP21 strain (Diaz et al., 2005).

Just before the challenge, stimulation of PBMC with VP21 produced 81 IFN-γ-SC/10^6 PBMC in G2, while the same stimulation only produced 21 IFN-γ-SC/10^6 PBMC in G1. After the challenge with VP21, G1 pigs became viremic (4/5) while G2 pigs did not. Therefore, V1 vaccine demonstrated a reduced ability to induce protective immune memory compared to V3 vaccine. The remaining pig in G1 that did not develop viremia had the higher IFN-γ-SC frequency at the day of challenge (45 IFN-γ-SC/10^6 PBMC). These results add a strong evidence for the role of the IFN-γ-SC in protection against PRRSV infection in piglets. Recently, Lowe et al. (2005) observed a strong correlation of cell-mediated immunity measured by IFN-γ-SC with protection against reproductive failure in sows of commercial herds as well. As discussed later, V1 had a higher ability to induce IL-10 secretion, and this could be have impaired the development of IFN-γ-SC. However, it cannot be ruled out that the adjuvant used with V3 had an influence on that observation although some authors denied the effect of adjuvants in PRRSV immunization (Meier et al., 2003).

Our results also showed that IFN-γ-SC frequencies were extremely low when V1 was used for in vitro stimulation. This was observed even in pigs vaccinated with V1. The impairment of IFN-γ-SC responses when V1 was used as a stimulus could be explained by the ability of V1 to induce the production of high levels of IL-10 in PBMC. Since this IL-10 production was seen in control pigs, it is reasonable to think that this was an intrinsic characteristic of the V1 strain and did not involve mechanisms of the adaptative immunity. Also, PBMC are thought to be non-permissive cells for PRRSV and therefore, the IL-10 release probably did not originate from viral replication in blood cells. The explanation for this fact remains unclear but a reasonable hypothesis is that IL-10 secretion was the result of the interaction of some of the envelope glycoproteins, the most variable part of the virus, with cellular receptors in monocytes or lymphocytes. However, this is only a hypothesis to be tested in further studies on PRRSV and immunity.

Regarding IL-10 secretion after recall PRRSV stimulation, it could be seen that this cytokine peaked between days 14 and 42 PV depending on the group and the strain (V3 or VP21) used. By day 63 PV, IL-10 was practically undetectable in cell culture supernatants. Disappearance of IL-10 correlated with the resolution of the viremia after vaccination. When G1 pigs were challenged with VP21 and developed viremia, IL-10 rose again. In a previous experiment (Diaz et al., 2005) we observed that in pigs experimentally infected with VP21, viremia and cessation of IL-10 production were also correlated. These facts suggest either that replication of the virus induces IL-10 or, alternatively, that IL-10 release sustains viral replication.

In the present experiment, as seen in control pigs, the potential of IL-10 secretion of PBMC changed over time. Thus, PHA stimulation produced some 700 pg/ml at 5 weeks of age and, from 8 weeks of age on, the same stimulus only yielded some 200–300 pg/ml. This fact indicated an influence of age on the ability to secrete IL-10. Interestingly, when PBMC from vaccinated pigs were stimulated in vitro with V1, a similar pattern was seen, but production of IL-10 was lower. From 42 days PV, V1 stimulation of PBMC from vaccinated pigs produced very low or undetectable IL-10 levels. Taken together, these observations can be interpreted as a virus-specific stimulation of PBMC that took place regardless of the immune status of the pig. The explanation for that is not fully clear but from 42 days PV on, frequencies of PRRSV-specific IFN-γ-SC rose. This suggests a role for the Th1/Th2 balance in this pattern.

Results for IL-2 and IL-4 responses did not show significant differences between control and vaccinated pigs using any of the viral strains. These data support the notion that some type of immunomodulation takes place in the course of the infection or vaccination (Royaee et al., 2004; Sipos et al., 2003).

Haptoglobin was only detected in sera of G1 animals after the challenge with VP21. Those animals were the only ones that developed viremia after the challenge. In our opinion, haptoglobin release was probably linked to the tissular damage caused by the wild virus but not by the replication of an

attenuated PRRSV strain. It is known that haptoglobin is related with IL-6 release in inflammatory processes (Asai et al., 1999).

In summary, the pattern of the evolution of both the humoral and the cellular immune responses after a PRRSV vaccination is very similar to that of a PRRSV infection in piglets (Diaz et al., 2005). Vaccine V1 had the lowest capacity to induce IFN-γ-SC and, in contrast, was able to stimulate a very high IL-10 release from PBMC. After the challenge with the virulent virus, the response of pigs vaccinated with V1 resembled that of an unvaccinated pig in terms of prevention of viremia. This observation leads to the notion that IFN-γ-SC most probably is the main factor in protecting against PRRSV infection as well as that IL-10 release may hamper the development of such IFN-γ-SC. Thus, the capacity of a given PRRSV strain to induce a strong cell-mediated immunity, measured as IFN-γ-SC, may be probably modulated by the IL-10 release (Chung and Chae, 2003; Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003; Diaz et al., 2005). Once the modulation of the immune response disappears, IFN-γ-SC frequencies rose and, when a given frequency is reached, the pig will be protected (Lowe et al., 2005). Nevertheless, this is only attained at late stage. On the other hand, since the ability to produce IL-10 may change with age, this host factor may also influence the outcome of the immunization or the infection.

In addition, we saw that the protection afforded by a given vaccine against a given PRRSV strain cannot be forecasted only by a global view of the genetic similarity between the vaccine virus and the challenge virus but, maybe, can be forecasted by examining the specific ability of a given viral strain to induce IFN-γ-SC and to modulate the immune responses. In consequence, the development of an effective vaccine for piglets should take into account the balance between IL-10 induction and the development of IFN-γ-SC. These data can be of critical importance to develop new and better PRRS vaccines.

Materials and methods

Animals and housing

Thirteen healthy 3-week-old Landrace pigs were randomly selected in a high health farm belonging to the Institut de Recerca i Tecnologia Agroalimentària (IRTA, Barcelona, Spain) that has historically been free of all major pig diseases including PRRS. Animals were transported to the experimental facilities, randomly divided in three groups (G1, G2 and C) and kept during 7 days to allow adaptation to the new conditions. Before the start of the experiment, pigs were examined for antibodies against porcine circovirus type 2, Aujeszky's disease virus, porcine parvovirus, swine influenza and *Mycoplasma hyopneumoniae*. All animals were seronegative for all of the abovementioned pathogens. Also, animals were confirmed to be free of PRRSV, as determined by ELISA (Herdcheck PRRS 2XR, IDEXX Laboratories, Westbrook ME, USA).

Vaccines and virus

Animals in group G1 (n = 5) were intramuscularly (IM) vaccinated with $10^{4.0}$ $TCID_{50}$ of a MLV made of one Spanish PRRSV strain (V1) using water as a vehicle (2 ml). G2 pigs (n = 5) received $10^{4.0}$ $TCID_{50}$ of a commercial MLV made of other European PRRSV strain (V3) (2 ml, IM) with an oil-in-water adjuvant. Vaccines were diluted with the vehicles recommended by each of the manufacturers. The remaining three pigs were kept as controls (C) and received 2 ml of sterile minimal essential medium (MEM) (Sigma, Alcobendas, Spain) as a placebo.

The virulent PRRSV VP21 strain used for the challenge was isolated in porcine alveolar macrophages (PAM) from sera of naturally infected pigs during an outbreak of PRRS affecting a breeding farm located in the north of Spain in December 1991. That outbreak was characterized by abortions, stillbirths and, later on, piglet mortality. The virus was shown to be of the European genotype by sequencing of ORFs 2 to 7. Viral stock was done by passage (n = 3) in PAM and titrated by means of the immunoperoxidase monolayer assay (IPMA) according to Wensvoort et al. (1991). Before to be used, the viral stock was checked for bacterial contamination and for the presence of other viruses.

At 63 days post-vaccination (PV), pigs in groups G1 and G2 were inoculated intranasally with 2 ml viral suspension containing 10^6 $TCID_{50}$/ml of PRRSV VP21 strain diluted in sterile MEM, whilst pigs in group C only received 2 ml of sterile MEM.

Virus sequencing and phylogenetic analysis

ORFs 2 to 7 of the vaccine viruses and challenge virus were amplified by PCR using specific primer pairs (Table 5). Sequencing was done using an ABI 3100 sequencer (Applied Biosystems). Sequences were formatted and translated to amino acid sequences. Alignments were done using ClustalW software. As a reference, Lelystad virus (LV) sequence (Genbank accession number M96262) was used. Nucleotide sequences were deposited at Genbank under accession numbers DQ009657-DQ009659 (ORF2); DQ009654-DQ009656 (ORF3); DQ067250, DQ064784, DQ064785 (ORF4); DQ009647, DQ064787, DQ064788 (ORF5), DQ009651-DQ009653 (ORF6) and DQ009648-DQ009650 (ORF7).

Samples

Blood samples were collected in duplicate (heparinized and siliconized blood-collecting tubes) immediately before vaccination and then at days 7, 14, 21, 28, 42, 63, 70 and 91 PV. Sera were used for PRRSV isolation and specific RT-nested PCR (nPCR), determination of humoral responses and evaluation of haptoglobin, IL-10 and transforming growth factor-beta (TGF-β) levels. Heparinized blood samples were used for hematological evaluation. Peripheral blood mononuclear cells (PBMC) were also obtained from heparinized blood samples and used for in vitro experiments.

Table 5
Oligonucleotides used for PCR amplification of different open reading frames (ORFs) from PRRSV with the respective annealing temperatures and the number of PCR cycles

Gene specificity	Primer[a]	Oligonucleotide sequences (5′–3′)	Annealing temperature (°C)	No. of cycles
ORF2	L	CTG GCA CAG AAT TGC AGG TA	55	45
	R	GCA CAC TGA TGA GCC ATT GT		
ORF3	L	ACA ATG GCT CAT CAG TGT GC	55	35
	R	TGA AGC CTT TCT CGC TCA TT		
ORF4	L	AGC GTG ACC ATG ATG AGT TG	55	39
	R	AAA AGC CAC CAG AAG CAA GA		
ORF5	L	TGA GGT GGG CTA CAA CCA TT	55	35
	R	AGG CTA GCA CGA GCT TTT GT		
ORF6	L	GTC CTC GAA GGG GTT AAA GC	55	35
	R	CTG TCC TCC CCT AGG TTG CT		
ORF7	L	GGC AAA CGA GCT GTT AAA CG	55	35
	R	AAT TTC GGT CAC ATG GTT CC		

[a] L, left or forward primer; R, right or reverse primer.

Detection of PRRSV

Serum samples were inoculated on MARC-145 and PAM cultures and incubated at 37 °C in 5% CO_2 for 90 min. Then sera were removed, and MEM was added. Cultures were incubated for three days at 37 °C in 5% CO_2. Infection of inoculated cells was determined by IPMA (Wensvoort et al., 1991).

Besides viral isolation, sera were examined by nPCR. Briefly, total RNA was extracted from 150 μl serum by using a commercial system (Nucleospin RNA virus; Macherey-Nagel, Düren, Germany). Total RNA was transcribed, and cDNA was used in a first PCR round directed to viral ORF5 (Mateu et al., 2003). This first-round PCR had a sensitivity of about 10^2 $TCID_{50}$/ml of serum. PCR products were used in an nPCR (forward primer, 5′-TCTTGCTTCTGGTGGCTTTT-3′; reverse primer, 5′-CATGTTTGATGGTGACGAGG-3′) that produced a 499-bp amplicon. Cycling parameters for both PCRs were 94 °C for 45 s; 55 °C for 45 s; 72 °C for 45 s for a total of 35 cycles. Under these conditions, the nPCR was considered to be able to detect <10 viral copies/ml of serum.

Hematology

Hematological cell counts were done using an automated system (Advia 120, Bayer) with protocols adapted to pig blood samples.

Humoral immune response

A commercially available ELISA (Herdchek PRRS 2XR, Idexx Laboratories) was used to measure PRRSV-specific antibodies. According to the manufacturer, sample to positive control (*S*/*P*) ratios of >0.4 were considered positive. PRRSV antibody titers were also determined in sera by using IPMA (Wensvoort et al., 1991), using MARC-145 cells infected with the PRRSV VP21 strain. Tests were done in duplicate.

NA were measured by the technique described by Jusa et al. (1996) and Yoon et al. (1994). Tests were done in triplicate by using whole serum, inactivated serum and serum plus 10% fresh guinea-pig complement. Briefly, 50 μl of each serum to be tested was diluted serially from 1/20 to 1/160 in cell culture medium. Dilutions were mixed with 50 μl viral suspension containing 200 $TCID_{50}$ of the PRRSV strain VP21 (with or without complement). Virus–serum mixtures were incubated for 1 h at 37 °C and then added to MARC-145 cultures in duplicate (96-well plates) and incubated for 3 days at 37 °C in 5% CO_2. Infection of cell cultures was revealed by using IPMA.

Isolation and culture of PBMC

PBMC were separated from whole blood by density-gradient centrifugation with Histopaque 1.077 (Sigma). For PBMC cultures, RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen, Spain), 1 mM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 5 mM 2-mercaptoethanol (Sigma), 50,000 IU/l penicillin (Invitrogen), 50 mg/l streptomycin (Invitrogen) and 50 mg/l gentamicin (Sigma) was used. Trypan blue stain was used to assess viability.

PRRSV-specific IFN-γ-SC

Frequencies of PRRSV-specific IFN-γ-SC in PBMC were analyzed by an ELISPOT assay using commercial mAbs (Swine IFN-γ Cytosets, Biosource Europe) according to a previously reported method (Díaz and Mateu, 2005). Briefly, ELISA plates (Costar 3590, Corning, USA) were coated overnight with 8.3 μg/ml IFN-γ capture antibody diluted in carbonate–bicarbonate buffer (pH 9.6). Plates were then washed and blocked for 1 h at 37 °C with 150 μl of PBS with 1% of bovine serum albumin. After removal of the blocking solution, 5×10^5 PBMC were dispensed per well (50 μl) and stimulated with V1, V3 or VP21 strains diluted in RPMI at a multiplicity of infection (MOI) of 0.01. After 20-h incubation at 37 °C in a 5% CO_2 atmosphere, cells were removed, and the biotinylated detection antibody was added at 2.5 μg/ml (50 μl) and incubated for 1 h at 37 °C. The reaction was revealed by sequential incubation of plates with streptavidin-peroxidase (1 h) and insoluble TMB blue (Calbiochem, Spain). Unstimulated cells and phytohemagglutinin (PHA)-stimulated controls (10 μg/ml) were also included.

To calculate the PRRSV-specific frequencies of IFN-γ-SC, counts of spots in unstimulated wells were subtracted from

counts in virus-stimulated wells. Frequencies of IFN-γ-SC were expressed as responding cells in 10^6 PBMC.

Cytokine (IL-2, IL-4, IL-10 and TGF-β) and haptoglobin ELISAs

PBMC were seeded at a density of 2×10^6 cells per well (250 μl) in 96-well plates and were mock stimulated or stimulated with V1, V3 or VP21 strains (MOI of 0.01) or PHA (10 μg/ml). After 24 h of incubation at 37 °C in 5% CO_2, cell culture supernatants were collected and frozen at −80 °C until needed. Capture ELISAs were performed as reported previously (Darwich et al., 2003; Díaz and Mateu, 2005) using commercial pairs of mAbs (Swine IL-2, IL-4, and IL-10 cytosets, Biosource Europe). The cut-off point of each ELISA was calculated as the mean optical density of negative controls plus three standard deviations. Cytokine concentrations were calculated using the linear regression formula from optical densities of the cytokine standards provided by the manufacturer. IL-10 and TGF-β were also measured in sera of experimental animals by using the abovementioned ELISAs. Detection limits for both cytokines were 32 pg/ml. Serum haptoglobin levels were determined by means of ELISA (Haptoglobin assay phase range, Tridelta, Ireland) according to the manufacturer's instructions.

Statistical analysis

The regression line of cytokine standards in ELISA was calculated by using SPSS v12.0 (SPSS Inc., Chicago, USA). Statistical comparisons between groups (Mann–Whitney/Kruskal–Wallis tests) were done by using Statsdirect v2.4.1. All tests done in the study, as well as the statistical analysis, were performed blind.

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References

Asai, T., Mori, M., Okada, M., Uruno, K., Yazawa, S., Shibata, I., 1999. Elevated serum haptoglobin in pigs infected with porcine reproductive and respiratory syndrome virus. Vet. Immunol. Immunopathol. 70, 143–148.

Balasuriya, U.B., MacLachlan, N.J., 2004. The immune response to equine arteritis virus: potential lessons for other arteriviruses. Vet. Immunol. Immunopathol. 102, 107–129.

Cafruny, W.A., Bradley, S.E., Rowland, R.R., 1999. Regulation of immune complexes during infection of mice with lactate dehydrogenase-elevating virus: studies with interferon-gamma gene knockout and tolerant mice. Viral. Immunol. 12, 163–173.

Christopher-Hennings, J., Nelson, E.A., Nelson, J.K., Benfield, D.A., 1997. Effects of a modified-live virus vaccine against porcine reproductive and respiratory syndrome in boars. Am. J. Vet. Res. 58, 40–45.

Chung, H.K., Chae, C., 2003. Expression of interleukin-10 and interleukin-12 in piglets experimentally infected with porcine reproductive and respiratory syndrome virus (PRRSV). J. Comp. Pathol. 129, 205–212.

Conzelmann, K.K., Visser, N., Van Woensel, P., Thiel, H.J., 1993. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. Virology 193, 329–339.

Darwich, L., Balasch, M., Plana-Duran, J., Segales, J., Domingo, M., Mateu, E., 2003. Cytokine profiles of peripheral blood mononuclear cells from pigs with postweaning multisystemic wasting syndrome in response to mitogen, superantigen or recall viral antigens. J. Gen. Virol. 84, 3453–3457.

Dee, S.A., Joo, H., 1997. Strategies to control PRRS: a summary of field and research experiences. Vet. Microbiol. 55, 347–353.

Díaz, I., Mateu, E., 2005. Use of ELISPOT and ELISA to evaluate IFN-γ, IL-10 and IL-4 responses in conventional pigs. Vet. Immunol. Immunopathol. 106, 107–112.

Diaz, I., Darwich, L., Pappaterra, G., Pujols, J., Mateu, E., 2005. Immune responses of pigs after experimental infection with a European strain of Porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 86, 1943–1951.

Forsberg, R., Oleksiewicz, M.B., Petersen, A.M., Hein, J., Botner, A., Storgaard, T., 2001. A molecular clock dates the common ancestor of European-type porcine reproductive and respiratory syndrome virus at more than 10 years before the emergence of disease. Virology 289, 174–179.

Grebennikova, T.V., Clouser, D.F., Vorwald, A.C., Musienko, M.I., Mengeling, W.L., Lager, K.M., Wesley, R.D., Biketov, S.F., Zaberezhny, A.D., Aliper, T.I., Nepoklonov E, A., 2004. Genomic characterization of virulent, attenuated, and revertant passages of a North American porcine reproductive and respiratory syndrome virus strain. Virology 321, 383–390.

Johnson, W., Roof, M., Vaughn, E., Christopher-Hennings, J., Johnson, C.R., Murtaugh, M.P., 2004. Pathogenic and humoral immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) are related to viral load in acute infection. Vet. Immunol. Immunopathol. 102, 233–247.

Jusa, E.R., Inaba, Y., Kouno, M., Hirose, O., Shibata, I., Kubota, M., Yasuhara, H., 1996. Slow-reacting and complement-requiring neutralizing antibody in swine infected with Porcine Reproductive and Respiratory Syndrome (PRRS) virus. J. Vet. Med. Sci. 58, 749–753.

Labarque, G., Van Gucht, S., Van Reeth, K., Nauwynck, H., Pensaert, M., 2003. Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines. Vet. Microbiol. 95, 187–197.

Labarque, G., Reeth, K.V., Nauwynck, H., Drexler, C., Van Gucht, S., Pensaert, M., 2004. Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. Vaccine 22, 4183–4190.

Lopez, O.J., Osorio, F.A., 2004. Role of neutralizing antibodies in PRRSV protective immunity. Vet. Immunol. Immunopathol. 102, 155–163.

Lowe, J.E., Husmann, R., Firkins, L.D., Zuckermann, F.A., Goldberg, T.L., 2005. Correlation of cell-mediated immunity against porcine reproductive and respiratory syndrome virus with protection against reproductive failure in sows during outbreaks of porcine reproductive and respiratory syndrome in commercial herds. J. Am. Vet. Med. Assoc. 226, 1707–1711.

Mateu, E., Martin, M., Vidal, D., 2003. Genetic diversity and phylogenetic analysis of glycoprotein 5 of European-type porcine reproductive and respiratory virus strains in Spain. J. Gen. Virol. 84, 529–534.

Meier, W.A., Galeota, J., Osorio, F.A., Husmann, R.J., Schnitzlein, W.M., Zuckermann, F.A., 2003. Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology 309, 18–31.

Meng, X.J., 2000. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet. Microbiol. 74, 309–329.

Meng, X.J., Paul, P.S., Halbur, P.G., Lum, M.A., 1995. Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. Arch. Virol. 140, 745–755.

Mengeling, W.L., Vorwald, A.C., Lager, K.M., Brockmeier, S.L., 1996. Comparison among strains of porcine reproductive and respiratory

syndrome virus for their ability to cause reproductive failure. Am. J. Vet. Res. 57, 834–839.

Mengeling, W.L., Lager, K.M., Vorwald, A.C., 1999. Safety and efficacy of vaccination of pregnant gilts against porcine reproductive and respiratory syndrome. Am. J. Vet. Res. 60, 796–801.

Mengeling, W.L., Lager, K.M., Vorwald, A.C., Clouser, D.F., 2003a. Comparative safety and efficacy of attenuated single-strain and multi-strain vaccines for porcine reproductive and respiratory syndromeVet. Microbiol. 93, 25–38.

Mengeling, W.L., Lager, K.M., Vorwald, A.C., Koehler, K.J., 2003b. Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. Vet. Microbiol. 93, 13–24.

Meulenberg, J.J., Hulst, M.M., de Meijer, E.J., Moonen, P.L., den Besten, A., de Kluyver, E.P., Wensvoort, G., Moormann, R.J., 1994. Lelystad virus belongs to a new virus family, comprising lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus. Arch. Virol. Suppl. 9, 441–448.

Meulenberg, J.J., Petersen den Besten, A., de Kluyver, E., van Nieuwstadt, A., Wensvoort, G., Moormann, R.J., 1997. Molecular characterization of Lelystad virus. Vet. Microbiol. 55, 197–202.

Mortensen, S., Stryhn, H., Sogaard, R., Boklund, A., Stark, K.D., Christensen, J., Willeberg, P., 2002. Risk factors for infection of sow herds with porcine reproductive and respiratory syndrome (PRRS) virus. Prev. Vet. Med. 53, 83–101.

Onyekaba, C.O., Harty, J.T., Even, C., Hu, B.G., Plagemann, P.G., 1989. Persistent infection of mice by lactate dehydrogenase-elevating virus: effects of immunosuppression on virus replication and antiviral immune responses. Virus Res. 14, 297–315.

Pesch, S., Meyer, C., Ohlinger, V.F., 2005. New insights into the genetic diversity of European porcine reproductive and respiratory syndrome virus (PRRSV). Vet. Microbiol. 107, 31–48.

Pirzadeh, B., Gagnon, C.A., Dea, S., 1998. Genomic and antigenic variations of porcine reproductive and respiratory syndrome virus major envelope GP5 glycoprotein. Can. J. Vet. Res. 62, 170–177.

Plana-Duran, J., Bastons, M., Urniza, A., Vayreda, M., Vila, X., Mane, H., 1997. Efficacy of an inactivated vaccine for prevention of reproductive failure induced by porcine reproductive and respiratory syndrome virus. Vet. Microbiol. 55, 361–370.

Royaee, A.R., Husmann, R.J., Dawson, H.D., Calzada-Nova, G., Schnitzlein, W.M., Zuckermann, F.A., Lunney, J.K., 2004. Deciphering the involvement of innate immune factors in the development of the host response to PRRSV vaccination. Vet. Immunol. Immunopathol. 102, 199–216.

Sipos, W., Duvigneau, C., Pietschmann, P., Holler, K., Hartl, R., Wahl, K., Steinborn, R., Gemeiner, M., Willheim, M., Schmoll, F., 2003. Parameters of humoral and cellular immunity following vaccination of pigs with a European modified-live strain of porcine reproductive and respiratory syndrome virus (PRRSV). Viral. Immunol. 16, 335–346.

Snijder, E.J., Meulenberg, J.J., 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79, 961–979.

Storgaard, T., Oleksiewicz, M., Botner, A., 1999. Examination of the selective pressures on a live PRRS vaccine virus. Arch. Virol. 144, 2389–2401.

Suradhat, S., Thanawongnuwech, R., 2003. Upregulation of interleukin-10 gene expression in the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 84, 2755–2760.

Suradhat, S., Thanawongnuwech, R., Poovorawan, Y., 2003. Upregulation of IL-10 gene expression in porcine peripheral blood mononuclear cells by porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 84, 453–459.

Van den Broek, M.F., Sporri, R., Even, C., Plagemann, P.G., Hanseler, E., Hengartner, H., Zinkernagel, R.M., 1997. Lactate dehydrogenase-elevating virus (LDV): lifelong coexistence of virus and LDV-specific immunity. J. Immunol. 159, 1585–1588.

Van Reeth, K., Labarque, G., Nauwynck, H., Pensaert, M., 1999. Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. Res. Vet. Sci. 67, 47–52.

Van Woensel, P.A., Liefkens, K., Demaret, S., 1998. Effect on viraemia of an American and a European serotype PRRSV vaccine after challenge with European wild-type strains of the virus. Vet. Rec. 142, 510–512.

Vezina, S.A., Loemba, H., Fournier, M., Dea, S., Archambault, D., 1996. Antibody production and blastogenic response in pigs experimentally infected with porcine reproductive and respiratory syndrome virus. Can. J. Vet. Res. 60, 94–99.

Wensvoort, G., Terpstra, C., Pol, J.M.A., 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Q. 13, 121–130.

Wu, W.H., Fang, Y., Rowland, R.R., Lawson, S.R., Christopher-Hennings, J., Yoon, K.J., Nelson, E.A., 2005. The 2b protein as a minor structural component of PRRSV. Virus Res. 114, 177–181.

Xiao, Z., Batista, L., Dee, S., Halbur, P., Murtaugh, M.P., 2004. The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. J. Virol. 78, 5923–5933.

Yoon, I.J., Goyal, S.M., Molitor, T.W., 1994. A modified serum neutralization test for the detection of antibody to porcine reproductive and respiratory syndrome virus in Swine sera. J. Vet. Diagn. Invest. 6, 289–292.

Yoon, K.J., Zimmerman, J.J., Swenson, S.L., McGinley, M.J., Eernisse, K.A., Brevik, A., Rhinehart, L.L., Frey, M.L., Hill, H.T., Platt, K.B., 1995. Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. J. Vet. Diagn. Invest. 7, 305–312.

Discusión

La distancia más corta entre dos puntos no es el camino que suelen seguir los discursos

Anónimo

4. DISCUSIÓN

Desde su aparición, el PRRS ha causado un importante impacto económico en el sector porcino. A causa de ello, las empresas farmacéuticas han desarrollado numerosas vacunas, basadas tanto en virus inactivados como en virus atenuados. Sin embargo, hasta la fecha ninguna vacuna ha podido aportar una protección total frente a la infección. Para poder mejorar la efectividad de dichas vacunas es imprescindible ampliar el escaso conocimiento que se tiene sobre los fenómenos que suceden en la interacción entre el PRRSV y el sistema inmunológico del cerdo. Así pues, el primer objetivo de esta tesis ha sido describir la respuesta inmune que se origina tras la infección por una cepa del genotipo europeo.

A partir de los resultados obtenidos en el primer experimento, parece evidente que la respuesta inmune frente a este virus se caracteriza por una elevada producción de anticuerpos, detectables por ELISA ya desde la primera semana post-infección en algunos animales; por una tardía respuesta de AN y por un desarrollo lento y errático de la respuesta celular medida como CP-IFN-γ específicas de virus. Con anterioridad, otros estudios han observado los mismos fenómenos en el transcurso de una infección por cepas del genotipo americano (Meier *et al.* 2003). Por tanto, y a pesar de las diferencias genéticas, el perfil de la respuesta de ambos genotipos es muy similar.

Tal y como han señalado varios autores, la respuesta humoral elevada y temprana detectable por ELISA no desempeñaría ninguna función en la protección frente a la infección. La detección de estos anticuerpos se considera más como una herramienta de diagnóstico que como un indicador de protección frente a la infección (Murtaugh *et al.,* 2002; López y Osorio, 2004). La incapacidad de estos anticuerpos en la función protectiva se ha demostrado en estudios *in vitro* (Yoon *et al.,* 1994) y en experimentos de protección pasiva (López y Osorio, 2004). Además, podría ser que estos anticuerpos no neutralizantes interviniesen en fenómenos de exacerbación de la infección en macrófagos (Yoon *et al.,* 1996).

Respecto a la respuesta humoral neutralizante, otros estudios han observado el mismo retraso en la producción de AN (Yoon *et al.,* 1994; Vezina *et al.,* 1996; Loemba *et al.,* 1996; Albina *et al.,* 1998b; Meier *et al.,* 2000 y 2003). Se ha sugerido la existencia de un epítopo de distracción como explicación a este fenómeno (Ostrowski *et al.,* 2002). Estos autores han descrito un

epítopo cercano al de neutralización, ambos localizados en la GP5, más inmunogénico y más accesible a la respuesta inmune que el epítopo neutralizante. Este epítopo de distracción sería inmunodominante y desviaría la respuesta humoral hacia él, provocando una aparición tardía de la respuesta de AN. Por otro lado, la región de la GP5 dónde se encuentra el epítopo neutralizante es una región altamente glicosilada; la unión de azúcares en esta zona del virus podría enmascarar el reconocimiento de este epítopo y alterar la respuesta de AN (Wissink *et al.,* 2003). Ninguno de estos dos hechos se han valorado con la cepa que hemos usado en la infección experimental, pero ambas hipótesis podrían explicar el retraso en la formación de AN. Este fenómeno permitiría al virus obtener un lapso de tiempo valioso para replicarse sin que el virión resultante pudiera ser neutralizado en el espacio extracelular.

Meier *et al.* (2003) demostraron que el desarrollo errático y tardío de las frecuencias de CP-IFN-γ es una característica específica del PRRSV también para las cepas americanas, ya que dicho desarrollo se estableció en magnitudes muy bajas e irregulares que no aumentaron de forma clara hasta las 20 semanas post-infección.

Con relación a las otras citoquinas analizadas en nuestros estudios, hay que destacar que no detectamos una producción evidente de IL-4, a pesar de la intensa respuesta humoral observada. Este hecho también se vió con cepas del genotipo americano (Royaee *et al.,* 2004). Tampoco hemos detectado una producción significativa de IL-2. Del mismo modo, Royaee *et al.* (2004) también observaron una falta de producción de IL-2, así como de otras citoquinas como la IL-12 y el IFN-α durante la infección experimental con cepas americanas. Así pues, la marcada respuesta Th2 podría deberse a la falta de citoquinas tipo Th1 durante la presentación y reconocimiento del antígeno. Todas estas observaciones, tanto las descritas por otros autores como las nuestras, describen una situación que perjudica el desarrollo inicial de la respuesta mediada por células, tal y como demuestra la evolución lenta e irregular de la frecuencia de CP-IFN-γ. En consecuencia, estos datos sugieren que existe algún tipo de inmunomodulación en el transcurso de la infección por el PRRSV (Molitor *et al,* 1997).

En el transcurso de la infección con la cepa española, y contrariamente a la escasa producción de IFN-γ y a la nula detección de IL-2 e IL-4, en los estudios *in vitro* detectamos una producción significativa de IL-10 en las fases iniciales. Este hecho coincidió con la fase de viremia. Trabajos anteriores observaron un aumento de la expresión del RNA mensajero de IL-10 en CMSP y en células procedentes de lavados traqueobronquiales pertenecientes a cerdos infectados con

cepas europeas o con cepas americanas (Suradhat *et al.,* 2003; Suradhat y Thanawongnuwech, 2003). Sin embargo, estos estudios habría que considerarlos como preliminares, pues el RNA mensajero de la IL-10 se expresa constitutivamente por muchos tipos celulares y su detección no implica la liberación de la proteína, debido a que ésta está sujeta a una fuerte regulación post-transcripcional siendo sólo secretada en presencia del estímulo adecuado (Redpath *et al.,* 2001). De hecho, Thanawongnuwech *et al.* (2001) no hallaron correlación entre los resultados de la RT-PCR y los de ELISA de IL-10 en cerdos infectados con el PRRSV. Según Suradhat *et al.* (2003) la elevada expresión de RNA mensajero de IL-10 estuvo asociada a virus viable y propusieron que la producción de IL-10 puede ser una de las posibles estrategias de evasión del PRRSV. Nuestro primer estudio aporta evidencias claras acerca de la capacidad del PRRSV para inducir la secreción de IL-10 en CMSP. El hecho de que esta capacidad se correlacionase en el tiempo con las fases de viremia sugiere que efectivamente la IL-10 puede desempeñar un papel en esta inmunomodulación; sin embargo no podemos afirmar si la secreción de IL-10 favorece la presencia de virus en sangre y la inhibición de las células productoras de IFN-γ, o bien es la replicación vírica la que induce la secreción de IL-10 que a su vez inhibe la respuesta de IFN-γ.

Durante la primera semana post-infección detectamos un aumento significativo de la población de linfocitos $CD8^+$ circulantes. Este aumento coincidió en el tiempo con el inicio de la detección de CP-IFN-γ, pero también con el pico de IL-10. Algunos autores han sugerido que la proliferación de los linfocitos $CD8^+$ en la infección del PRRSV no está asociada a una expansión clonal específica del virus, sino que podría tratarse de una proliferación inespecífica como resultado de la acción de alguna citoquina o de otros mediadores liberados en el curso de la infección (Shimizu *et al.,* 1996; Albina *et al.,* 1998b; Labarque *et al.,* 2000; Samsom *et al.,* 2000). Así pues, no podemos descartar que la proliferación de estos linfocitos sea fruto de la acción inespecífica de la IL-10. De hecho, en otros modelos se ha descrito que esta citoquina promueve la proliferación de linfocitos $CD8^+$ (Groux *et al.,* 1998; Rowbottom *et al.,* 1999) y que la activación de los linfocitos en presencia de IL-10 puede inducir anergia (Groux *et al.,* 1996), por lo que el aumento de $CD8^+$ podría estar desvinculado a la producción de IFN-γ. El papel de la IL-10 como inductor y factor de mantenimiento de estados de baja respuesta inmune o anergia (Becker *et al.,* 1994; Sundstedt *et al.,* 1997) ha sido sugerido para explicar los fenómenos observados en el transcurso de algunas infecciones víricas, como el HIV o el Friend virus del ratón (Iwashiro *et al.,* 2001; Dittmer *et al.,* 2004). En este sentido, se ha demostrado que la significación biológica de la proliferación de CD8+ en la respuesta frente al PRRSV resulta cuanto menos incierta. Lohse *et al.* (2004) observaron que provocando una depleción temporal de esta subpoblación celular en

cerdos y posteriormente infectándolos con el PRRSV, éstos no mostraban una exacerbación de la sintomatología ni ningún cambio evidente en la resolución de la infección, por lo que los resultados obtenidos eran equiparables a los cerdos control no tratados e infectados.

En nuestro estudio hemos podido observar que la viremia cesó en ausencia de AN. Meier *et al.* (2003) también han podido observar este hecho con una cepa americana, lo que sugiere que el papel de los AN en la eliminación del virus durante el curso de la infección es, probablemente, limitado, a pesar de que algunos autores han observado una clara correlación entre la aparición de AN frente a la GP5 y el cese de la viremia (Pirzadeh y Dea, 1997; Murtaugh *et al.,* 2003). De todos modos, nuestras observaciones no nos permiten establecer ningún juicio de valor sobre su papel en la protección de un animal que ya tuviera AN antes de infectarse. Así, López y Osorio (2004) han demostrado que la transferencia pasiva de anticuerpos neutralizantes puede otorgar protección frente a la infección. No obstante, esta protección, medida en cerdas gestantes como la inexistencia de transmisión transplacentaria del PRRSV a la descendencia y la prevención del fallo reproductivo, se obtuvo únicamente con unos niveles de AN muy elevados que raramente aparecen tras una infección (Osorio *et al.,* 2002). Curiosamente, los mismos autores han señalado que la transferencia pasiva de AN a estos mismos niveles no parece otorgar inmunidad esterilizante a cerdos jóvenes (Osorio y López, 2004). En nuestro caso, los AN sólo aparecieron a partir de los dos meses post-infección y nunca en todos los animales. En definitiva, no podemos desvincular los AN del desarrollo de la obtención de una inmunidad protectiva, pero creemos que resulta claro que tras la infección la viremia puede resolverse en ausencia de AN detectables, por lo que serían otros los componentes de la respuesta inmune encargados de eliminar al virus.

El objetivo de nuestro segundo experimento fue valorar qué parámetros eran los importantes en la inmunidad protectiva inducida por los PRRSV atenuados. Una de las primeras y más importantes diferencias que hemos podido observar en el transcurso de este experimento es que los cerdos vacunados presentaban periodos de viremia más largos que los cerdos infectados en nuestro primer trabajo. Concretamente, tras la vacunación y de un modo intermitente, podían hallarse animales virémicos hasta las siete semanas post-vacunación, en contraste con los 14 días que duraba la viremia en los animales infectados en el primer estudio. Creemos que este hecho podría estar influenciado por la edad de los animales, más jóvenes en este segundo trabajo, pero las largas viremias descritas con cualquiera de las dos cepas vacunales evidencian una situación que puede ser potencialmente peligrosa, ya que el virus se

está replicando y por tanto es posible su excreción y posible transmisión a otros animales. Este hecho refleja una situación totalmente indeseable a nivel práctico. La excreción y la transmisión (Botner *et al.,* 1997; Madsen *et al.,* 1998; Storgaard *et al.,* 1999; Nielsen *et al.,* 2001) e incluso la reversión a virulencia de cepas atenuadas del PRRSV (Botner *et al.,* 1997; Mengeling *et al.,* 1999; Nielsen *et al.,* 2001 y 2002; Opriessnig *et al.,* 2002) es un fenómeno ampliamente descrito. Por otro lado, nuestros datos muestran que la extensión de la viremia no debe interpretarse como un equivalente de la virulencia del virus; de hecho, no se observó ningún signo clínico en los animales vacunados. En este sentido, Xiao *et al.* (2004) han propuesto que la replicación del virus ocurre mientras existen macrófagos permisivos y por lo tanto una cepa atenuada, con un ciclo citolítico menos intenso, podría replicarse durante más tiempo.

Respecto a la respuesta inmune tras la vacunación, ésta se desarrolló de forma muy parecida a la que se observó tras la infección experimental de nuestro primer estudio. Meier *et al.* (2003) observaron también este mismo desarrollo comparando cepas de campo y cepas atenuadas del genotipo americano. Por tanto, parece que los mecanismos que participan en el desarrollo de la respuesta inmune tras la vacunación o la infección son similares independientemente del genotipo. Contrariamente, no ocurren los mismos fenómenos con la inmunización de los cerdos con vacuna inactivada, en cuyo caso los títulos de anticuerpos son muy bajos y la respuesta mediada por células es escasa (Meier *et al.,* 2003; Nilubol *et al.,* 2004). Esto sugiere que los mecanismos inmunes que se desencadenan con la inoculación de una cepa viva de PRRSV, ya sea atenuada o virulenta, son similares, mientras que la respuesta es distinta con un virus inactivado.

Tal y como sucedía con el desarrollo en la respuesta a la infección, los resultados de este segundo trabajo sugieren que la inmunidad protectiva conferida por las cepas atenuadas vacunales no está relacionada con la producción de AN, ya que no se detectaron en ningún animal antes del desafío. Por otro lado, después del desafío con la cepa VP21, no se detectó el virus en la sangre de los cerdos que tenían los niveles de CP-IFN-γ más altos; es decir, los cinco cerdos del grupo vacunal G2 y uno del G1. Por tanto, los cerdos con las frecuencias de CP-IFN-γ más elevadas estuvieron protegidos frente al desarrollo de viremia. Respecto al primer estudio de esta tesis, existió un primer pico de CP-IFN-γ en la segunda semana post-infección, coincidiendo con la desaparición del virus en sangre. Todos estos datos indican que la participación de los AN en la protección fue escasa o nula, mientras que las frecuencias de CP-IFN-γ tuvieron una intensa participación en la protección frente al desafío y, quizás, en la

resolución de la viremia tras la infección. Recientemente, Lowe *et al.* (2005) han observado también una fuerte correlación entre la respuesta celular medida como la frecuencia de CP-IFN-γ y la protección, en este caso frente al fallo reproductivo causado por el PRRSV en cerdas de granjas comerciales.

Curiosamente, en nuestro estudio la protección frente al desafío inducida por la vacuna heteróloga (V3), cuya similitud con la cepa de desafío VP21 osciló entre el 92.3 y el 98.6% según la ORF analizada, fue mayor que la inducida por la vacuna hómologa (V1). La vacuna V1, con una similitud con la cepa VP21 entre el 98.3 y el 99.7%, tuvo una menor capacidad de promover la respuesta de CP-IFN-γ. Sin embargo, esta cepa estimuló la producción de elevadas cantidades de IL-10. Este fenómeno queda claramente reflejado en el hecho de que inducía la liberación de IL-10 incluso en CMSP de animales inmunitariamente vírgenes. Todo ello sugiere que la protección frente al desafío puede estar modulada por la IL-10 y su efecto sobre la evolución de las frecuencias de CP-IFN-γ. Así, es posible obtener una protección heteróloga si se alcanzan unos niveles de CP-IFN-γ elevados o, por el contrario, no proteger frente a una cepa muy similar a la vacunal si ésta induce una elevada producción de IL-10. Probablemente, cuando la modulación que ejerce el PRRSV sobre la respuesta inmune desaparece, las frecuencias de CP-IFN-γ aumentarían y el cerdo quedaría protegido. En consecuencia, a partir de estos resultados podemos concluir que el desarrollo de una vacuna efectiva tendría que considerar no sólo la similitud genética con las cepas de campo, sino también las propiedades inmunes de dicha cepa, concretamente su habilidad de inducir CP-IFN-γ y su potencial inmunomodulador, probablemente debido a la capacidad de inducir la producción de IL-10.

En definitiva, las observaciones descritas en los dos trabajos de la presente tesis junto a las descritas por otros autores, demuestran que existe un desequilibrio en las respuestas inmune frente al PRRSV, tanto en cepas atenuadas como de campo y tanto en cepas del genotipo europeo como del americano. Para explicar este desajuste inmunitario se han planteado dos hipótesis que, en nuestra opinión, no tienen porque ser excluyentes entre sí y que plantean un nuevo modelo de patogenia. La primera hipótesis considera como el factor clave la liberación de citoquinas reguladoras en las primeras fases de la infección (Suradhat *et al.,* 2003; Royaee *et al.,* 2004), mientras que la segunda aboga por una participación secundaria de la respuesta inmune y por una mayor repercusión de las dinámicas poblacionales de macrófagos permisivos en la resolución de la infección (Xiao *et al.,* 2004).

Algunos patógenos están implicados en estrategias que facilitan su persistencia en el hospedador mediante la manipulación de la respuesta inmune (Redpath *et al.,* 2001). Se considera que la actividad de los linfocitos encargados de llevar a cabo la respuesta Th1, su maduración y la producción de IFN-γ, así como la actividad de macrófagos y la presentación de antígeno, podría estar alterada por citoquinas secretadas en las primeras fases de algunas infecciones, como la IL-10 (Murray, 1999). A partir de los análisis realizados en nuestros estudios, la detección de una elevada producción de IL-10 puede considerarse como una posible causa que explique la falta de desarrollo de la respuesta adaptativa celular.

La producción de IL-10 puede estar asociada a los monocitos/macrófagos o a los linfocitos T e incluso B como un mecanismo normal de la respuesta inmune frente a un patógeno con el objeto de regular una actividad inflamatoria excesiva y por tanto dañina. Sin embargo, la actividad de la IL-10 no sólo se limita a la función antiinflamatoria, sino que en los últimos años se le ha asignado la etiqueta de citoquina reguladora de varias citoquinas, algunas de ellas esenciales para el correcto desarrollo de las respuestas adaptativas celulares (Moore *et al.,* 2001; Mills, 2004). Como ya hemos comentado, se han detectado bajos niveles o incluso prácticamente inexistentes de IL-2, IL-12 (Royaee *et al.,* 2004), IFN-α (Albina *et al.,* 1998a; Van Reeth *et al.,* 1999; Meier *et al.,* 2003; Royaee *et al.,* 2004) o TNF-α (Van Reeth *et al.,* 1999) tras la infección por el PRRSV. Paralelamente, la IL-10 también es un potente factor de proliferación y diferenciación de los linfocitos B, así como del mantenimiento de su actividad (Rousset *et al.,* 1992; Itoh y Hirohata, 1995; Hu y Insel, 1999). Tras la infección por el PRRSV, se ha descrito una expansión policlonal de linfocitos B en ganglios linfáticos (Lamontagne *et al.,* 2001). Los ganglios linfáticos son, junto al pulmón y las tonsilas, una de las localizaciones donde se concentra la mayor cantidad de virus, y por tanto, podría también concentrase una mayor cantidad de IL-10.

Se desconoce la población celular encargada de la liberación de la IL-10, ya sea como respuesta a la presencia de una fuerte respuesta inflamatoria o por la estimulación directa del PRRSV, pero teniendo en cuenta infecciones de otras especies pueden proponerse algunas hipótesis. Los estudios realizados en modelos de infección en el ratón y en el hombre han demostrado que algunos patógenos son capaces de explotar el uso de la IL-10 como vía de escape de la respuesta inmune a partir de su inducción en monocitos/macrófagos o a partir de linfocitos reguladores. En las infecciones por el virus de la inmunodeficiencia humana (HIV) y por el virus de la Hepatitis C se han descrito asociaciones directas entre ciertas proteínas de estos virus y la

producción de IL-10 por parte de los monocitos de las CMSP (Schols y DeClerq, 1996; Brady *et al.,* 2003). En el caso del HIV se ha sugerido que la IL-10 liberada es capaz de inducir anergia en las CMSP y por tanto interferir en su funcionalidad. Un hecho que podría apoyar la hipótesis de la participación de monocitos/macrófagos en la liberación de IL-10 durante la infección por el PRRSV es la detección de la haptoglobina. Esta proteína se detectó a niveles significativos en los animales infectados de nuestro primer estudio y, en el segundo estudio, en los animales vacunados que desarrollaron estados de viremia tras el desafío. Se ha descrito que la liberación de haptoglobina está relacionada con la secreción de citoquinas anti-inflamatorias, como la IL-10, mediante la interacción con el receptor CD163 de monocitos/macrófagos (Moestrup y Moller, 2004; Philippidis *et al.,* 2004).

Otra de las mayores fuentes productoras de IL-10 son los linfocitos T reguladores (Tr), naturales o inducidos (McGuirk y Mills, 2002; Damoiseaux, 2006). Los estados de anergia o de respuesta baja hacia un antígeno concreto también pueden ser mediados por la acción de los linfocitos Tr (Doetze *et al.,* 2000; McGuirk y Mills, 2002). El marcaje y la diferenciación de estos tipos celulares están en proceso de mejora en la especie humana y en el ratón, pero es un campo prácticamente inexplorado en el porcino. Los Tr naturales se identifican por las moléculas de superficie CD4+/CD25+ y el *forkhead/winged helix transcriptional factor 3* (FOXP3) (Coffer y Burgering, 2004; Mills, 2004). En nuestro primer estudio, no existió un aumento significativo de los marcadores $CD4^+$ o $CD25^+$ en las poblaciones circulantes. En cuanto al FOXP3, cuya expresión en la superficie de los Tr naturales está estrechamente asociada a su funcionalidad (Hori y Sakagucki, 2004), existen anticuerpos monoclonales para su marcaje en humana y ratón, pero su uso en células porcinas arrojan resultados muy variables y no útiles hasta la fecha (datos propios no publicados). De los subtipos de Tr inducidos, es decir específicos de antígeno, el mayor productor de IL-10 son los Tr1 (McGuirk y Mills, 2002; Mills y McGuirk, 2004). Los Tr1, por acción de la IL-10, inhiben la producción de los TNF e IL-12 de macrófagos, la proliferación de células Th1 y Th2 y la liberación por parte de estas células de IFN-γ e IL-4 (McGuirk y Mills, 2002), por lo que la participación de células Tr1 se antoja, junto a la participación de monocitos/macrófagos, como una de las hipótesis más plausibles para explicar las alteraciones de la respuesta frente al PRRSV asociadas a la producción de IL-10.

Debido a la falta de desarrollo de una respuesta eficaz tanto en la infección del PRRSV como de otros arterivirus, se ha sugerido que la respuesta inmune tendría un papel secundario en la superación de la infección (Xiao et al., 2004). Dentro de la familia Arteriviridae, el LDV es el virus

más cercano al PRRSV (Cavanagh, 1997). Este virus del ratón infecta a los macrófagos, causa largas viremias e induce una pobre y lenta respuesta de AN y linfocitos T citotóxicos (Chen *et al.,* 1994; van den Broek *et al.,* 1997). En este caso, parece evidente que el papel de la respuesta inmune es secundario, ya que tras la infección la duración de la viremia no se ve afectada en los ratones tratados con sustancias que reducen su capacidad de respuesta inmunológica (Onyekaba *et al.,* 1989). Además, los ratones *knock-out* nu/nu, que tienen un desarrollo anómalo del timo y por tanto unos linfocitos T funcionalmente deficientes, tienen el mismo nivel de viremia que los ratones normales (Onyekaba *et al.,* 1989). En consecuencia, se ha sugerido que la infección del LDV estaría controlada principalmente por la reducción en la población de los macrófagos permisivos (Onyekaba *et al.,* 1989; Plagemann y Moennig, 1992). En el caso del PRRSV, se sabe que sólo entre el 2-5% de los macrófagos son permisivos a la infección (Duan *et al.,* 1997; Xiao *et al.,* 2004) y por tanto la eliminación de éstos limitaría la extensión de la infección. Si la dinámica de la infección ocurriera exclusivamente de este modo, puede interpretarse que el descenso de la frecuencia de CP-IFN-γ atiende más a la redistribución de las células circulantes como respuesta a la persistencia del virus en los tejidos linfoides, según porcentaje de macrófagos permisivos, que a una acción de las citoquinas reguladoras. En este caso, la variabilidad observada en las respuestas estaría relacionada con el porcentaje de macrófagos permisivos de cada individuo. A partir de este modelo, la protección frente a una re-infección homóloga se explicaría por la falta de macrófagos permisivos. Sin embargo, parece evidente que la respuesta celular, medida como frecuencia de CP-IFN-γ específicas, aporta protección frente a la infección, a pesar de que los niveles necesarios para ello no aparezcan, en el mejor de los casos, antes de los dos meses post-vacunación. Todo ello sugiere que, probablemente, los mecanismos involucrados pueden ser múltiples e incluir tanto la modulación de la respuesta inmune como la dinámica de infección en los macrófagos. Incluso sería posible que diferentes cepas regulasen de forma distinta la respuesta inmune e interactuasen de un modo diferente con los macrófagos.

A partir de nuestros estudios, una de las posibles explicaciones del retraso de la respuesta celular es la participación de citoquinas reguladoras. Como crítica a estos resultados hay que tener en cuenta que la sangre periférica contiene tan sólo el 2% del total de los linfocitos T (Westermann y Pabst, 1992) y que ni la replicación del virus ni la presentación del antígeno se realiza en el compartimiento vascular. Por tanto, futuros estudios deberían ampliar nuestro conocimiento sobre las citoquinas liberadas en los lugares típicos de replicación y acantonamiento de este virus, es decir órganos linfoides, sobre todo tonsilas, y pulmón. Debería

conocerse también el origen de la producción de las citoquinas reguladoras con el objeto de poder entender mejor cómo, dónde y con qué células es capaz de interactuar el PRRSV. Algunos de los estudios más inmediatos que planteamos incluyen el uso de anticuerpos neutralizantes de IL-10 en los cultivos o la co-estimulación *in vitro* con dos cepas de CMSP de cerdos vacunados, una de ellas V1, para demostrar que la IL-10 liberada está inhibiendo la producción de IFN-γ. De todos modos, dada la elevada variabilidad de este virus, desconocemos si la capacidad intrínseca de la cepa V1 para inducir la liberación de IL-10 es un hecho puntual o no. Por tanto, para poder establecer una relación entre la secuencia genética y la inducción de IL-10 deberían caracterizarse en este sentido muchas más cepas. La respuesta inmune es un sistema redundante con múltiples puntos de inducción y de regulación de un mismo mecanismo, por lo que la liberación de IL-10 podría incluso inducirse por diferentes vías según la cepa. Por este mismo motivo, además de los linfocitos Tr y los monocitos/macrófagos, tampoco debería descartarse la participación de células NK reguladoras, linfocitos Tγδ reguladores o incluso linfocitos CD8+ reguladores, poblaciones descritas muy recientemente en otras especies (Bach, 2003; Damoiseaux, 2006). Por otro lado, no podemos descartar que la resolución de la infección esté también asociada a la dinámica de las poblaciones de macrófagos permisivos, por lo que debería mejorarse la caracterización de estos macrófagos y su interacción con el PRRSV. Respecto a la eficacia de las vacunas atenuadas, debemos concluir que la protección otorgada por una vacuna no se ciñe solamente a su semejanza con la cepa infectiva, sino también a sus propiedades inmunológicas. Así, una de las posibles vías para la mejora de las vacunas debería incluir un estudio profundo de las propiedades inmunológicas de las cepas a usar y de la potenciación de las respuestas Th1 con adyuvantes específicos. No podemos pues descartar que una vacuna atenuada, inactivada o incluso de subunidades o de DNA pueda ser efectiva siempre y cuando ésta incluya los epítopos inductores de AN y de CP-IFN-γ y evite en su composición los epítopos, secuencias o proteínas inductoras de respuestas indeseables, como la producción de anticuerpos no neutralizantes o de IL-10.

Conclusiones

Una conclusión es el lugar donde llegaste cansado de pensar

Anónimo

5. CONCLUSIONES

1. El perfil de la respuesta inmune de los animales infectados con una cepa española del virus del síndrome reproductivo y respiratorio porcino se caracteriza por una ausencia de IL-2 e IL-4, un desarrollo de las células productoras de IFN-γ lento e irregular y una producción tardía de anticuerpos neutralizantes. A pesar de las diferencias genéticas entre genotipos, este perfil es similar al descrito para las infecciones por las cepas del genotipo americano.

2. En el curso de la infección de los lechones con una cepa española del virus del síndrome reproductivo y respiratorio porcino, la fase de viremia se correlaciona con la respuesta de IL-10. Por otra parte, el desarrollo de células productoras de IFN-γ no alcanza niveles significativos hasta el declive de la IL-10. Estas observaciones sugieren que o bien la secreción de IL-10 favorece la presencia de virus en sangre y la inhibición de las células productoras de IFN-γ, o bien es la replicación vírica la que induce la secreción de IL-10 que a su vez inhibe la respuesta de IFN-γ.

3. En los animales infectados, la viremia cesó en ausencia de anticuerpos neutralizantes. Este hecho sugiere que en la infección natural los anticuerpos neutralizantes desempeñan un papel secundario y deben ser otros los mecanismos involucrados en la contención de la infección.

4. El perfil de la respuesta inmune de los animales vacunados con las vacunas estudiadas es muy parecido al que se observa tras la infección con una cepa española de campo del virus del síndrome reproductivo y respiratorio porcino, excepto para el desarrollo de anticuerpos neutralizantes que no se detectan en los dos primeros meses tras la vacunación. Este hecho sugiere que los mecanismos que intervienen en el desarrollo de la inmunidad tras la vacunación o la infección son similares.

5. Los animales vacunados que habían alcanzado frecuencias de células productoras de IFN-γ específicas de virus superiores a 45 por millón de células mononucleares de sangre periférica en el momento del desafío, estuvieron protegidos del desarrollo de viremia pese a carecer de anticuerpos neutralizantes. Este hecho indica que las frecuencias de células productoras de IFN-γ pueden ser indicadores de protección.

6. En nuestro estudio, la protección inducida por la vacuna V3 frente al desafío heterólogo fue mayor que la inducida por la vacuna V1 frente al desafío homólogo. La vacuna V1 poseía una gran capacidad de inducir IL-10 aun en animales inmunitariamente vírgenes. En las condiciones de nuestro estudio, estas observaciones indicarían que dentro de un mismo genotipo, la protección frente al desafío homólogo o heterólogo puede estar modulada por la interacción entre la IL-10 inducida por una cepa concreta y el desarrollo de células productoras de IFN-γ, de tal modo que cuando se alcanzan frecuencias de células productoras de IFN-γ elevadas se obtiene incluso protección heteróloga.

Resumen

En la realidad no ocurre nada que corresponda rigurosamente a la lógica

Friedrich Nietzsche

6. RESUMEN

El Síndrome Reproductivo y Respiratorio Porcino (PRRS) es una enfermedad de gran impacto en la cabaña porcina mundial. La enfermedad se caracteriza por provocar en los animales jóvenes un cuadro básicamente respiratorio, acompañado de anorexia y fiebre, mientras que en las reproductoras puede ocasionar un cuadro reproductivo caracterizado por la presencia de abortos tardíos, fetos momificados, partos prematuros y lechones nacidos muertos.

El agente etiológico de esta enfermedad, conocido como virus del PRRS (PRRSV) o arterivirus porcino, se caracteriza por tener una elevada variabilidad genética. Se han descrito dos genotipos, el americano (cepa de referencia ATCC-VR-2332) y el europeo (cepa de referencia virus del Lelystad) cuya similitud en algunas de las ORFs (*Open Reading Frames* o fragmentos de lectura abierta) que los componen no supera el 60%. Además, existe una elevada diversidad genética de cepas dentro de cada genotipo.

Debido a las graves consecuencias que acarrea esta infección se han comercializado vacunas atenuadas e inactivadas, tanto de origen europeo como de origen americano. Los resultados obtenidos con el uso de estas vacunas arrojan cuanto menos ciertas dudas sobre la eficacia de las mismas. Una de las mayores razones por la que no se han podido desarrollar vacunas más eficientes es la falta de un conocimiento profundo de los sucesos inmunológicos que acontecen tras la infección.

Otros estudios, realizados principalmente con cepas del genotipo americano, han sugerido que la respuesta inmune frente a este virus es anómala; con una elevada producción de anticuerpos no neutralizantes y una respuesta celular medida por células productoras de interferon-γ (CP-IFN-γ) baja y de evolución lenta. Así pues, el propósito de esta tesis doctoral ha sido caracterizar de forma global la respuesta inmune del cerdo frente a cepas del PRRSV del genotipo europeo; en primer lugar durante la infección de lechones con una cepa de campo española, y en segundo lugar tras la vacunación con vacunas atenuadas, evaluando también en este último caso la protección que ofrecen dichas vacunas frente a un desafío homólogo o heterólogo.

En el primer trabajo de esta tesis se ha querido realizar una descripción integral de la respuesta inmune que se origina tras la infección de los cerdos con una cepa perteneciente al genotipo europeo. Así mismo, se ha pretendido relacionar los hallazgos observados con otros estudios anteriores de carácter global realizados con cepas americanas y con otros más de carácter parcial realizados con cepas europeas. Los hallazgos más significativos de la respuesta de los cerdos infectados experimentalmente con una cepa española son: una ausencia de producción de IL-2 e IL-4, la aparición temprana de anticuerpos no neutralizantes en contraposición a una respuesta neutralizante inconstante y tardía, y un desarrollo lento e irregular de células productoras de IFN-γ (CP-IFN-γ) específicas de virus. Los resultados obtenidos sugieren que, en primera instancia, el papel que desarrollan los anticuerpos neutralizantes durante la infección debe considerarse limitado, ya que estos no son necesarios para la eliminación del virus de la sangre. Por otro lado, para la cepa española estudiada en esta tesis, una de las hipótesis que planteamos como explicación a la lenta evolución de la respuesta celular es la producción de IL-10 en las primeras fases de la infección, fenómeno que es coincidente con la viremia. Todos los análisis llevados a cabo en este primer trabajo demuestran que, a pesar de las diferencias genéticas existentes entre los dos genotipos, la respuesta inmune que se desarrolla tras la infección es prácticamente igual, caracterizándose ésta por presentar una marcada polarización Th2 potente y no resolutiva y por una evolución lenta y errática de la respuesta Th1.

En el segundo estudio se pretendió evaluar la respuesta inmune desarrollada tras la vacunación de lechones con dos vacunas atenuadas de genotipo europeo, así como la protección que éstas otorgaban frente a un desafío homólogo o heterólogo. Un primer hallazgo que a nivel práctico resulta interesante remarcar es que los cerdos vacunados presentaban periodos de viremia más largos que los cerdos infectados en el primer estudio. Aunque no puede descartarse que este fenómeno esté sesgado por efecto de la edad de los cerdos, los animales de este segundo trabajo eran más jóvenes, las largas viremias descritas con cualquiera de las dos cepas vacunales denotan una situación donde el virus está replicándose durante largo tiempo y dónde se posibilita la excreción de éste y por tanto la infección y la hipotética reversión de virulencia. Respecto a la respuesta inmune, ésta se desarrolló de forma muy parecida a la que se observó tras la infección. Sin embargo, en este caso la respuesta humoral neutralizante no apareció antes de los 2 meses post-vacunación y sólo en algunos animales tiempo después del desafío. Los fenómenos observados en este estudio sugieren que son las CP-IFN-γ el parámetro inmune que debería considerarse como marcador de protección, ya que sólo los animales que alcanzaban una magnitud mínima de CP-IFN-γ en el momento del desafío quedaron protegidos,

independientemente de la vacuna que les fue administrada. Curiosamente la vacuna que aportó un mayor grado de protección fue la vacuna heteróloga (V3) a la cepa de desafío (VP21). La vacuna homóloga (V1) poseía una elevada capacidad de inducir la producción de IL-10 incluso en las células de los animales no vacunados ni infectados, por lo que éste hecho se muestra como una de las explicaciones más plausibles del retraso de la respuesta celular observado en el grupo de lechones vacunados con la cepa V1. Todo ello sugiere que no sólo la homología del virus vacunal en relación a la cepa infectante es importante para la protección, sino que también hay que tener en cuenta el tiempo transcurrido tras la vacunación y las propiedades inmunológicas inherentes de la cepa vacunal.

Todos estos hallazgos demuestran que la respuesta inmune de los cerdos frente a la infección y frente a la vacunación con cepas atenuadas del PRRSV del genotipo europeo es muy parecida entre sí y, a la vez, muy semejante con la descrita frente a cepas de campo y atenuadas del genotipo americano. Así pues, la respuesta inmune frente al PRRSV puede considerarse anómala ya que se caracteriza por un déficit de los marcadores de la respuesta celular y una ineficiente respuesta humoral. Respecto a las vacunas estudiadas en esta tesis, consideramos que los animales vacunados pueden estar protegidos incluso frente a cepas heterólogas si los niveles de CP-IFN-γ son suficientes, y que en algunos casos la evolución de dichos niveles podría estar asociada a la producción de IL-10; factor que debe considerarse dependiente de cepa.

Anexo

¡Empieza tu día con una sonrisa! Verás lo divertido que es ir andando por ahí desentonando con todo el mundo.

Mafalda

7. ANEXO

En el anexo de la presente tesis se muestra una compilación de otros artículos y aportaciones a congresos internacionales publicados durante la realización de la misma y que están relacionados con el PRRSV o con la evaluación de la respuesta inmunológica del cerdo.

7.1 Otras publicaciones

Use of ELISPOT and ELISA to evaluate IFN-g, IL-10 and IL-4 responses in conventional pigs
Veterinary Immunology and Immunopathology, 106:107–112

En este estudio se llevó a cabo la estandarización de las pruebas de ELISPOT para la determinación de las citoquinas IFN-γ (marcadora de la respuesta Th1), IL-4 (marcadora de la respuesta Th2) e IL-10 (citoquina reguladora) en células mononucleares de sangre periférica estimuladas con PHA. Además, los resultados obtenidos se compararon con los obtenidos mediante ELISA apartir de sobrenadantes. Las conclusiones más significativas extraídas a partir de este estudio fueron: a) aumento de las células secretoras de IFN-γ con la edad; b) mantenimiento del número de células secretoras de IL-4 a niveles muy inferiores al resto de citoquinas analizadas; c) mantenimiento de un número constante de células secretoras de IL-10 y presencia de una elevada secreción espontánea; d) la correlación entre ambas técnicas sólo pudo demostrarse con la IL-10; los bajos niveles de IL-4 no permitían su detección mediante ELISA, mientras que la diferencia de tamaños de los *spots* de IFN-γ demostraban que existen subpoblaciones celulares con diferente capacidad secretora de esta citoquina. Todos estos datos, además de poder utilizarse como referencia para estudios futuros, señalan que los resultados obtenidos mediante ELISA y ELISPOT no tienen necesariamente que correlacionarse.

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Use of ELISPOT and ELISA to evaluate IFN-γ, IL-10 and IL-4 responses in conventional pigs

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Abstract

ELISPOT and ELISA were standardised for pig interferon-γ (IFN-γ), interleukin-10 (IL-10) and interleukin-4 (IL-4) with the aim to study the evolution of the immune response in conventional pigs from birth to 6 months of age and also to compare results of both techniques. Five pigs were bled at 1, 6, 9, 12 and 22 weeks of age and peripheral blood mononuclear cells (PBMC) were stimulated with phytohemagglutinin. The frequencies of cytokine secreting cells (CSC) and the levels of secreted cytokines were compared. For IFN-γ the mean of CSC increased with age ($p < 0.05$) from an average of $486/10^6$ PBMC at first week of age to $1256/10^6$ PBMC at 22 weeks of age. No correlation was found between the number of IFN-γ CSC and the cytokine levels obtained by ELISA. For IL-10, frequencies of CSC did not increase with age of pigs, having a low of $315/10^6$ PBMC at first week of age and a high of $1485/10^6$ PBMC at six weeks. Comparison of ELISA and ELISPOT results for IL-10 showed a certain degree of correlation ($r = 0.74$; $p < 0.05$). Spontaneous secretion was observed in unstimulated cultures. For IL-4, frequencies of CSC were low (50–$70/10^6$ PBMC). In this case, comparison of ELISA and ELISPOT could not be done because cytokine levels in culture supernatants were often below the detection limit of the IL-4 ELISA. All these values can serve as a reference for future studies and also, our observations suggest that ELISPOT and ELISA should be carefully interpreted and do not necessarily correlate.

Keywords: Pig; Cytokines; ELISPOT; ELISA

1. Introduction

The role of cytokines in regulation and modulation of the immune response has been widely studied since the Th1/Th2 paradigm was postulated. This paradigm proposes that, after priming, T helper cells polarize in two distinct subsets (Th1 and Th2) that can be distinguished by their cytokine expression and functional abilities (Mosmann et al., 1986; Mosmann and Coffman, 1989). Although reality seems to be more complex than a simple dichotomous polarization, this paradigm is useful to understand the adaptative immune response. Thus, IL-12 and IFN-γ are considered to be key cytokines in Th1 profiles

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and serve as indicators of a predominance of cell-mediated responses, whereas IL-4 participates in the Th2 polarisation and its secretion suggests a predominance of humoral responses. Regarding IL-10, it is thought that it can be an element participating in both type of immune responses having a regulatory effect (McGuirk and Mills, 2002). Therefore, the measurement of cytokine production is essential to understand the adaptative immune response.

The enzyme-linked immunospot assay (ELISPOT) is a powerful technique for the quantitation of cytokine responses (Czerkinsky et al., 1988). However, its use has been scarce in veterinary immunology because of the lack of adequate mAbs. In the case of swine, ELISPOT has been used for quantitation of IFN-γ and IFN-α (Zuckermann et al., 1998; Foss et al., 2002; Nowacki et al., 1993; Splichal et al., 1997) but few other cytokines have been examined by this technique. The aim of this work was to standardize ELISPOT to study the evolution of IFN-γ, IL-10 and IL-4 cytokine-secreting cells (CSC) in pigs from birth to six months of age. Also, correlation of frequencies of CSC with the amount secreted cytokines as determined by ELISA was done to assess the equivalence between these two techniques.

2. Materials and methods

Five 1-week-old conventional piglets from the same litter were randomly selected in a conventional farm and were bled at 1, 6, 9, 12 and 22 weeks of age. Blood samples were collected in heparinized tubes by jugular venipuncture and PBMC were isolated by density gradient centrifugation using Histopaque® 1.077 (Sigma, Alcobendas, Spain). Washed PBMC of each animal were resuspended in RPMI-1640 supplemented with 10% foetal calf serum (Invitrogen, Barcelona, Spain), 1 mM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 5 mM 2-mercaptoethanol (Sigma), 50,000 IU/l penicillin 1 (Invitrogen), 50 mg/l streptomycin (Invitrogen) and 50 mg/l gentamicin (Sigma). In parallel, microtiter plates (Costar 3590, Corning Incorporated, USA) had been coated overnight with optimal concentrations (determined in previous experiment) of IFN-γ, IL-10 or IL-4 capture antibodies (Swine IFN-γ, IL-10 and IL-4 Cytosets™ kits, Cat. no. CSC4034, CSC0104 and CSC1284, respectively; Biosource Europe, Nivelles, Belgium) diluted in carbonate–bicarbonate buffer, pH 9.6 (IFN-γ and IL-10) or PBS, pH 7.2 (IL-4), washed with PBS and blocked for 1h at 37 °C with 100 μl of

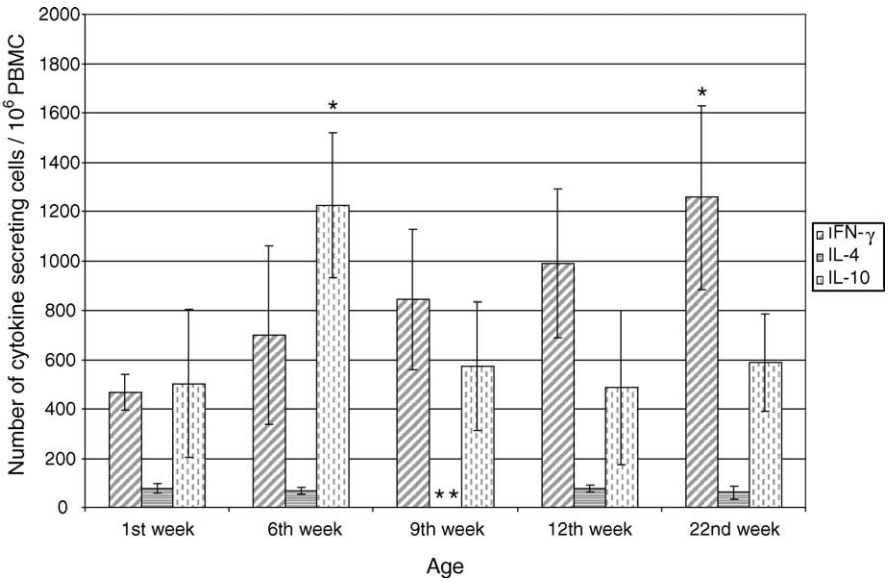

Fig. 1. Evolution during first six months of life of the mean number of IFN-γ, IL-4 and IL-10 secreting cells in pig peripheral blood mononuclear cells (five pigs). Cells were stimulated in vitro with PHA. Results are represented as the average and standard deviation. The mean number of IFN-γ secreting cells increased as determined by the linearity test ($p < 0.05$). *$p < 0.05$; **IL-4 not done.

PBS supplemented with 1% BSA. After removing of blocking solution, 50 μl/well of PBMC were dispensed in coated wells at densities ranging from 2.5×10^4 to 2×10^5 cells/well. Then, cell cultures were stimulated with PHA at 10 μg/ml or were mock-stimulated with plain culture medium. Parallel cultures performed under the same conditions were done in uncoated plates (Costar 3590, Corning Incorporated) to have cell culture supernatants to be analysed in ELISA.

After 20 h of incubation at 37 °C in 5% CO_2 atmosphere, cells were removed and cell culture supernatants were stored at −80 °C until further examination. In coated plates, the adequate biotinilated detection antibody (anti-IFN-γ, IL-10 or IL-4, provided by the manufacturer) was added to each well and incubated 1 h at 37 °C. After washing, reaction was revealed by sequential incubation of plates with streptavidin-peroxidase (provided by the manufacturer) at 0.5 μg/ml for IFN-γ and IL-10 and at 2.5 μg/ml for IL-4 (1 h, 37 °C), and insoluble TMB blue membrane (Calbiochem, Barcelona, Spain). Frequencies of CSC were expressed as the number of responding cells per million of PBMC. For this count, the number of spots in unstimulated cultures was subtracted from the count of PHA-stimulated cultures. Cytokine levels in cell culture supernatants were evaluated by ELISA using the same antibody pairs than in ELISPOT and adding soluble ABTS (Calbiochem) as a substrate. Cytokine concentrations were calculated from a regression equation obtained from optical densities of IFN-γ, IL-10 and IL-4 standards provided with the antibody pairs.

In ELISPOT, optimal concentrations of capture antibodies were 8.3, 15.1 and 9.6 μg/ml for IFN-γ, IL-10 and IL-4, respectively. Detection antibodies were used optimally at 2.5, 2.5 and 5.0 μg/ml. Concentration of mAbs for ELISA was two to three times lower. Using the standards provided by the manufacturer of mAbs, detection limits in ELISA were 62, 32 and 32 pg/ml for IFN-γ, IL-10 and IL-4, respectively.

Statistical analysis of the evolution of CSC over time was done by means of the linearity test. Comparisons between the amounts of secreted cytokines and the number of CSC were done by a regression analysis and Mann–Whitney test was used to compare results among weeks. All statistical analysis was performed using Statsdirect®.

3. Results and discussion

Regarding ELISPOT results, frequencies of IFN-γ CSC changed over time. Thus, mean number of IFN-γ CSC increased with age ($p < 0.05$) from $486 \pm 73/10^6$ PBMC at one week of age to $1256 \pm 373/10^6$ PBMC at 22 weeks of age (Fig. 1). With these counts, optimal density of cells/well was determined to be between 2.5 and 5.0×10^4. At all ages, spots showed a wide variation in size (Fig. 2a). In ELISA, IFN-γ concentrations varied from 200 to 2000 pg/ml but no clear correlation was seen between the number of spots and the yield of cytokine in cell culture supernatants.

Although the experiments were conducted without separation of T cell subsets, the increase of IFN-γ CSC with age could be attributable to the increase of CD4/CD8 double positive T cells (DP). In early life of pigs, DP lymphocytes account for less than 2% of the total PBMC pool but later on this value can increase up to 60% of total PBMC (Zuckermann, 1999). This DP subset is comprised of memory and effector cells among which a substantial part of the IFN-γ CSC phenotype can be found (Zuckermann and Husmann, 1996; Zuckermann, 1999; Rodriguez-Carreño et al., 2002). Under natural farm conditions, it would be expected that antigenic stimuli would induce such an increase of DP cells. Interestingly, frequencies of IFN-γ CSC did not correlate with the amount of secreted cytokine. This result disagrees partially with the observations of Favre et al. (1997) with mouse spleen cells. Those authors found a strong correlation between ELISPOT and ELISA for in vitro cultured cells. Also, the average amount of IFN-γ secreted per cell changed with age being highest at 1 week of age and lowest at 22 weeks (Table 1). This result could correspond to variations of the relative proportions of T cell subsets. It is known that in young pigs γδ T-cells account for a large proportion of blood PBMC. As pig grows, γδ cells decrease and DP cells increase (Yang and Parkhouse, 1996; Zuckermann, 1999). According to Rodriguez-Carreño et al. (2002) pig γδ T-cell display a high capacity to secrete IFN-γ and this may explain why the highest amounts of IFN-γ secretion per cell was seen at one week of age.

For IL-10, optimal cell density for ELISPOT was similar to that of IFN-γ. The mean number of IL-10 CSC was fairly constant over time (about $540 \pm 40/$

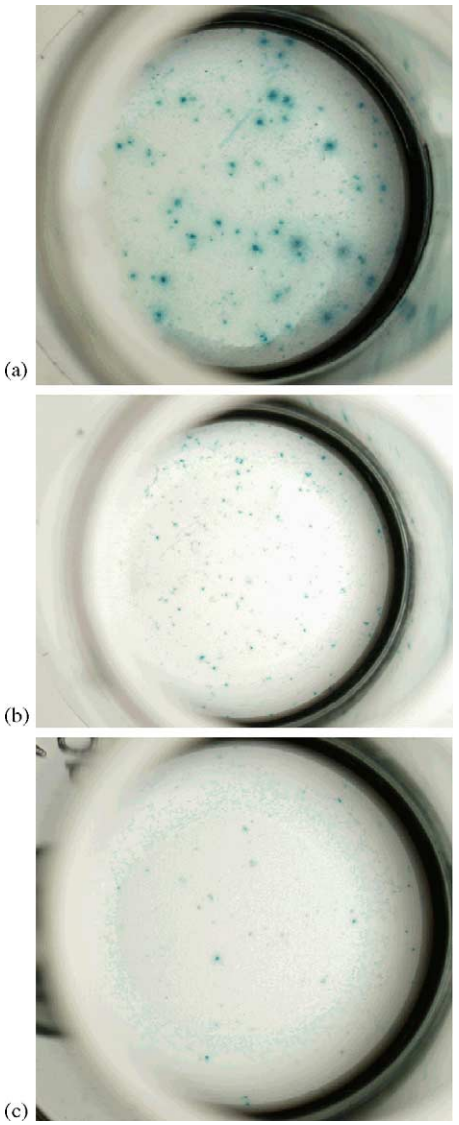

Fig. 2. ELISPOT images for peripheral blood mononuclear cells stimulated with PHA. (a) IFN-γ, (b) IL-10 and (c) IL-4. Notice the similar size of IL-10 and IL-4 spots compared to IFN-γ ones.

10^6 PBMC), except at six weeks of age where an increase was noticed ($1224 \pm 292/10^6$ PBMC; $p < 0.05$) (Fig. 1). This increase in IL-10 CSC at six weeks of age could reflect the impact of weaning upon the immune system as seen with several cytokines in pig and mouse gut (Pie et al., 2004; Vazquez et al., 2000).

Table 1
Amounts of IFN-γ, IL-10 and IL-4 secreted per cell after in vitro stimulation of porcine peripheral blood mononuclear cells with PHA

Age (weeks)	Amount of cytokine secreted per cell (pg) (mean ± standard deviation)		
	IFN-γ	IL-10	IL-4
1	4.03 ± 1.01^{a}	0.13 ± 0.07	0.05 ± 0.09
6	0.25 ± 0.07^{b}	0.07 ± 0.07	<0.01
9	0.56 ± 0.27^{b}	0.10 ± 0.03	<0.01
12	0.21 ± 0.20^{bc}	0.10 ± 0.04	0.03 ± 0.07
22	0.11 ± 0.09^{c}	0.10 ± 0.06	0.04 ± 0.1

Values with different superscript letters (a–c) are significantly different ($p < 0.05$). Results were calculated by dividing the concentration of each cytokine in cell culture supernatants (ELISA) by the number of cytokine secreting cells (ELISPOT).

Interestingly, spontaneous secretion of IL-10 was observed in unstimulated wells in all animals at all ages (Table 2). In contrast to IFN-γ CSC, size of IL-10 spots was small and with little differences among them (Fig. 2b). When IL-10 levels were determined by ELISA, we found a significant correlation between the amount of secreted cytokine and the number of spots ($r = 0.742$; $CI_{95\%} = 0.476$–0.884; $p < 0.05$) (Fig. 3). IL-10 levels in cell culture supernatants from ELISPOT positive cultures ranged from 32 to 100 pg/ml, being the amount of cytokine secreted per cell constant at all ages (Table 1).

The constant frequency of IL-10 CSC suggested either that under natural conditions there is no significant increase of the cell subsets producing this cytokine or, that IL-10 was mainly produced by monocytes or other cells without need of a recall or mitogen stimulation. Previous studies indicated that IL-10 can be constitutively expressed in several cell

Table 2
Spontaneous number of cytokine secreting cells per 10^6 of peripheral blood mononuclear cells obtained from unstimulated cultures of 1–22-weeks-old pigs

Age (weeks)	Spontaneous cytokine secreting cells/10^6 PBMC (mean ± standard deviation)		
	IFN-γ	IL-10	IL-4
1	0 ± 0	27 ± 21	0 ± 0
6	4 ± 9	152 ± 145	2 ± 4
9	0 ± 0	12 ± 11	0 ± 0
12	4 ± 5	34 ± 39	0 ± 0
22	0 ± 0	404 ± 331	0 ± 0

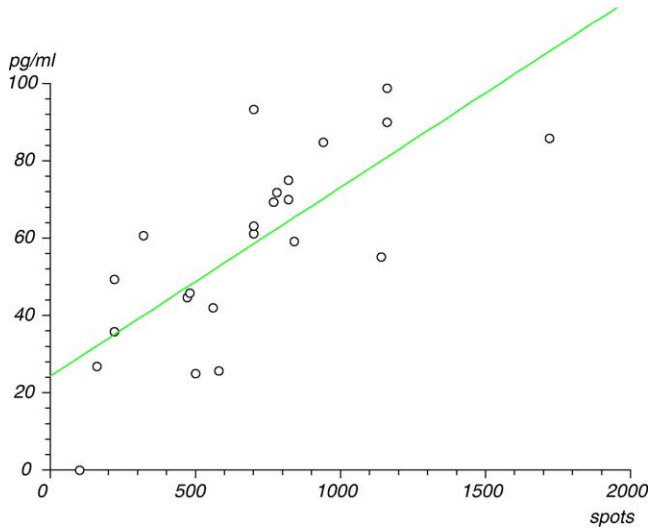

Fig. 3. Correlation between the amounts of secreted IL-10 (pg/ml) with the number of IL-10 secreting cells/10^6 peripheral blood mononuclear cells (spots) for five pigs. The value of correlation coefficient was 0.742 ($CI_{95\%}$ = 0.476–0.884; $p < 0.05$).

types (Moore et al., 2001). Guerkov et al. (2003) suggested that when using ELISPOT, the IL-10 secretory activity of monocytes or B-cells can mask antigen-specific T-cell responses. To rule out the participation of monocytes in spontaneous IL-10 release, we also prepared monocyte cultures (from 1×10^4 up to 5×10^4 cells/well) stimulated with LPS or mock-stimulated. No response was obtained for cells cultured with plain cell culture medium, while LPS-stimulated cultures yielded IL-10 CSC frequencies of about 100–200 spots/10^6 monocytes, indicating that spontaneous secretion attributable to monocytes, which normally represented between 5 and 10% of total PBMC preparations, accounted only for a small fraction of total IL-10 spots in PBMC. Also, to assess whether this secretion was ex novo or not, parallel cultures of PBMC and monocytes were treated with cycloheximide. This treatment completely inhibited IL-10 release. With this data, it is difficult to determine the origin of the spontaneous IL-10 production and by now we have no explanation for that. However, the small size of some of the spots points out that probably cells other than memory T-lymphocytes, maybe B-cells, were participating in these observations (Guerkov et al., 2003).

The mean number of IL-4 secreting cells was constant at about $70 \pm 20/10^6$ PBMC (Fig. 1). These frequencies raised optimal cell densities up to at least 10^5 cells/well. In general, IFN-γ CSC and IL-10 CSC levels were approximately 5–20-fold higher than IL-4 CSC. These low levels of response compared to other cytokines are consistent with previous observations that showed similar results for human spleen cells (Favre et al., 1997). However in pigs, Nuntrapasert et al. (2004) using a combination of Con A, Ionomycin, PMA and PHA reached frequencies of IL-4 CSC about $75/10^4$ PBMC. We do not know the reason for such a discrepancy of results but, maybe, it could be attributable to the fact that they did not use the same reagents.

IL-4 levels in culture supernatants of PHA-stimulated cells were low or even undetectable by ELISA. For this reason, the correlation between the amount of IL-4 secreted and the frequencies of IL-4 CSC could not be calculated. This fact could indicate a higher sensitivity of IL-4 ELISPOT compared to ELISA (Favre et al., 1997; Tanguay and Killion, 1994). Besides, other researchers suggested that in vitro consumption of IL-4 in cell cultures can also contribute to this discrepancy of results between ELISA and ELISPOT (Ewen and Baca-Estrada, 2001).

The present study shows that both ELISA and ELISPOT are useful to measure the cytokine responses of pig PBMC. However, interpretation of results should be carefully done since negative results in

ELISA can correspond to significant responses in ELISPOT (IL-4) or because frequencies of responding cells not necessarily correspond with the intensity of cytokine secretion. Also, for IFN-γ, frequencies of CSC may vary with age, a fact that implies the need to adapt densities of cells in the ELISPOT assay to make it readable. Furthermore, for IL-10 the participation of cells other than T-lymphocytes is suspected and this should be further investigated to gain insight on how results should be interpreted.

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References

Czerkinsky, C., Andersson, G., Ekre, H.P., Nilsson, L.A., Klareskog, L., Ouchterlony, O., 1988. Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. J. Immunol. Methods 110, 29–36.

Ewen, C., Baca-Estrada, M.E., 2001. Evaluation of interleukin-4 concentration by ELISA is influenced by the consumption of IL-4 by cultured cells. J. Interferon Cytokine Res. 21, 39–43.

Favre, N., Bordmann, G., Rudin, W., 1997. Comparison of cytokine measurements using ELISA. ELISPOT and semi-quantitative RT-PCR. J. Immunol. Methods 204, 57–66.

Foss, D.L., Zilliox, M.J., Meier, W., Zuckermann, F., Murtaugh, M.P., 2002. Adjuvant danger signals increase the immune response to porcine reproductive and respiratory syndrome virus. Viral Immunol. 15, 557–566.

Guerkov, R.E., Targoni, O.S., Kreher, C.R., Boehm, B.O., Herrera, M.T., Tary-Lehmann, M., Lehmann, P.V., Schwander, S.K., 2003. Detection of low-frequency antigen-specific IL-10-producing CD4(+) T cells via ELISPOT in PBMC: cognate vs. nonspecific production of the cytokine. J. Immunol. Methods 279, 111–121.

McGuirk, P., Mills, K.H., 2002. Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. Trends Immunol. 23, 450–455.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., O'Garra, A., 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19, 683–765.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L., 1986. Two types of murine helper T cell clone: I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136, 2348–2357.

Mosmann, T.R., Coffman, R.L., 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 7, 145–173.

Nowacki, W., Cederblad, B., Renard, C., La Bonnardiere, C., Charley, B., 1993. Age-related increase of porcine natural interferon alpha producing cell frequency and of interferon yield per cell. Vet. Immunol. Immunopathol. 37, 113–122.

Nuntrapasert, A., Mori, Y., Fujita, K., Yoneda, M., Miura, R., Tsukiyama-Kohara, K., Kai, C., 2004. Characterization of specific antibodies and the establishment of sandwich ELISA and ELISPOT systems for swine IL-4. Comp. Immunol. Microbiol. Infect. Dis. 27, 457–470.

Pie, S., Lalles, J.P., Blazy, F., Laffitte, J., Seve, B., Oswald, I.P., 2004. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. J. Nutr. 134, 641–647.

Rodriguez-Carreño, M.P., López-Fuertes, L., Revilla, C., Ezquerra, F., Alonso, F., Domínguez, J., 2002. Phenotypic characterization of porcine IFN-γ-producing lymphocytes by flow cytometry. J. Immunol. Methods 259, 171–179.

Splichal, I., Rehakova, Z., Sinkora, M., Sinkora, J., Trebichavsky, I., Laude, H., Charley, B., 1997. In vivo study of interferon-alpha-secreting cells in pig foetal lymphohaematopoietic organs following in utero TGEV coronavirus injection. Res. Immunol. 148, 247–256.

Tanguay, S., Killion, J.J., 1994. Direct comparison of ELISPOT and ELISA-based assays for detection of individual cytokine-secreting cells. Lymphokine Cytokine Res. 13, 259–263.

Vazquez, E., Gil, A., Garcia-Olivares, E., Rueda, R., 2000. Weaning induces an increase in the number of specific cytokine-secreting intestinal lymphocytes in mice. Cytokine 12, 1267–1270.

Yang, H., Parkhouse, R.M., 1996. Phenotypic classification of porcine lymphocyte subpopulation in blood and limphoid tissues. Immunology 89, 76–83.

Zuckermann, F.A., Husmann, R.J., 1996. The human and swine CD4/CD8 double positive T lymphocyte. In: Tumbleson, M.E., Schook, L.B. (Eds.), Advances in Swine Biomedical Research. Plenum Press, New York, pp. 331–343.

Zuckermann, F.A., 1999. Extrathymic CD4/CD8 double positive T cells. Vet. Immunol. Immunopathol. 72, 55–66.

Zuckermann, F.A., Husmann, R.J., Schwartz, R., Brandt, J., Mateu de Antonio, E., Martin, S., 1998. Interleukin-12 enhances the virus-specific interferon gamma responses of pigs to an inactivated pseudorabies virus vaccine. Vet. Immunol. Immunopathol. 63, 57–67.

Evolution of ORF5 of Spanish porcine reproductive and respiratory syndrome virus strains from 1991 to 2005

Virus Research, 115:198–206

El objetivo de este estudio era determinar qué cambios genéticos se habían dado en las cepas españolas del PRRSV desde su aparición. Para ello se analizaron las secuencias de las ORF5 de 18 cepas comprendidas entre 1991 y 1995 y de 46 de entre 2000 y 2005; se determinaron los aminoácidos que codificaban y los cambios potenciales que podían llevarse a cabo en la GP5. Las conclusiones principales de este trabajo fueron: 1) la similitud con la cepa de referencia del genotipo europeo (virus del Lelystad) ha decrecido desde el 95.5% (cepas hasta 1995), hasta el 89.5% (cepas del 2000 al 2005); 2) la mayoría de puntos de selección se hallan en las regiones transmembrana de la GP5; 3) los datos obtenidos sugieren que el PRRSV aun se está adaptando a las células de su hospedador.

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Short communication

Evolution of ORF5 of Spanish porcine reproductive and respiratory syndrome virus strains from 1991 to 2005

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Abstract

ORF5 sequences of porcine reproductive and respiratory syndrome virus (PRRSV) were analysed to determine genetic diversity, codon usage, positive and negative selection sites and potential changes in the predicted glycoprotein 5 (GP5). A hypothetical GP5 containing all selected sites was constructed to determine its characteristics. These sequences corresponded to isolates obtained 10 years apart (1991–1995, 18 strains) and a second set ($n = 46$) from 2000 to 2005. Similarity to Lelystad virus (LV) decreased from 95.5% in 1991–1995 to 89.5% in 2000–2005. Three highly variable regions were found in ORF5. Codon usage was different in both sets for leucine, glutamine, serine and proline. Thus, 2000–2005 sequences used codons more similar to those present in highly expressed pig genes compared to the 1991–1995 set. Twenty four sites of positive selection and 20 sites of negative selection were found in GP5, most of them in transmembrane regions. Additional glycosylation in N37 of GP5 was common in 2000–2005 but some sequences lack a glycosylation site in N46. The hypothetical GP5 was only 88.1% similar to LV and was less hydrophobic. Taking together these results suggest that PRRSV is still adapting to pig cells.

Keywords: Porcine reproductive and respiratory virus; ORF5; Genetic diversity; Codon usage

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the genus *Arterivirus*. Two different genotypes are currently known, the European one and the American one. Genetic similarity between both genotypes ranges from 50 to 70% depending on the open reading frame (ORF) examined (Meng et al., 1994). PRRSV genome is composed of nine ORFs of which ORF1a and 1b encode the viral polymerase, ORFs 2–6 encode envelope and membrane proteins (called GP2a, GP2b, GP3, GP4, GP5 and M, respectively) and ORF7 encodes the non-glycosylated viral nucleocapsid protein (Snijder and Meulenberg, 1998; Wu et al., 2005). Among the envelope proteins, GP5 seems to be one of the key viral structures. It is thought that attachment and entry to the target cells is mediated by GP5 or GP5-M heterodimers (Snijder et al., 2003). In addition, the neutralisation epitope of PRRSV is located in the middle of the GP5 ectodomain (Gonin et al., 1999; Ostrowski et al., 2002; Plagemann, 2004a,b).

From early studies, it became evident that ORF5 was one of the most variable regions in the PRRSV genome although other parts also show a considerable degree of variability as occurs in ORF3 and in non-structural protein 2 (nsp2) (Fang et al., 2004; Oleksiewicz et al., 2000). ORF5 heterogeneity was initially reported for American strains but nowadays is evident that European isolates are diverse as well (Forsberg et al., 2002; Mateu et al., 2003; Pesch et al., 2005; Pirzadeh et al., 1998). Genetic variability observed in ORF5 is consistent with the well known fact that RNA-polymerases of RNA viruses have a relatively poor fidelity (Castro et al., 2005) and with the notion that selective pressures act favouring viral variants better fitted for spread and persistence in the target hosts. As GP5 is exposed in the viral envelope, participates in viral attachment to cells and contains a neutralisation epitope, it is a potential target for these selective pressures. Interestingly, the adaptative sites in GP5 seem not to be restricted to the known B-epitopes but also to other regions (Hanada et al., 2005).

In the present study, a large set of ORF5 sequences from Spanish PRRSV strains obtained 10 years apart were analysed

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to determine the changes in ORF5 and GP5 and to figure out the potential impact of those changes.

ORF5 sequences of Spanish PRRSV strains were randomly selected from isolates available in our laboratory obtained from sera of pigs of Spanish epidemiologically unrelated commercial farms or were retrieved from Spanish sequences deposited at Genbank. For PRRSV isolates, viral RNA was extracted, amplified by PCR and sequenced as described before (Mateu et al., 2003). The final set of sequences included the Lelystad virus (LV) (Genbank accession number M96262), the first Spanish isolate from 1991 (Genbank accession number X92942), a second Spanish isolate from 1991 (strain CReSA-VP21, GenBank accession number DQ009647), 15 isolates from 1991 to 1995 (Suárez et al., 1996) and 46 unrelated isolates retrieved in our laboratory from 2000 to 2005 (Genbank accession numbers AF495499–AF495502, AF495504–AF5521, DQ009625–DQ009646). For comparative purposes, one set of 16 non Spanish European-type ORF5 sequences representing strains isolated in the period 1991–1995 in several countries of Europe were also analysed. This set comprised sequences from Belgium ($n = 2$), Denmark ($n = 2$), France ($n = 2$), Germany ($n = 2$), The Netherlands ($n = 3$), Poland ($n = 2$) and United Kingdom ($n = 1$) (GenBank accession numbers: AY035900–AY035903, AY035918, AY035919, AY035921, AY035922, AY035926, AY035927, AF378799, U40696, U40696 and M96262).

ORF5 sequences were initially aligned using ClustalW (Thompson et al., 1994) and a similarity matrix was constructed. Alignments were retrieved using Bio-edit software v.7.0.5 (available at http://www.mb.mahidol.ac.th/Downloads/Mol-Bio/Bioedit/Bioedit.htm) and entropy plots were constructed to determine genetic variability in ORF5. Entropy was calculated as $H(\mathrm{l}) = -\sum f(b, l) \times \ln(f(b, l))$ where entropy H(l) is equal to the summatory of $f(b,l)$, namely the frequency at which a given residue (b) is found at a given position (l), multiplied by the neperian logarithm of $f(b,l)$. With this formula, the higher the value of H(l), the higher the variability at a given position. Lelystad virus was used as a reference for alignments.

Codon usage for each set of sequences was analysed using GCUA software v.1.0 (available at http://bioinf.may.ie). In a first step, relative codon usage was calculated for each set of sequences by means of the synonymous codon usage measures (RSCU) and taking into account the effective number of codons (ENC) in the gene. Then, a correspondence analysis (CA) was done in order to determine trends in the variation of codon usage. A linear regression analysis was used to evaluate correlation between codon usage bias and nucleotide composition. p-Values lower than 0.05 were considered to be significant. To ascertain the possible significance of changes in codon usage, 10 sequences of genes highly expressed in pigs were also analysed. Genes included were: creatin kinase (Genbank accession number AY754869); interferon-beta (NM_001003923), pyruvate dehydrogenase (X52990), myosin heavy chain (NM_214136); haptoglobin (AF492467), hemoglobin epsilon (NM_214447); plasminogen activator (AF364605), albumin (NM_00100528), alpha amylase (AF064742) and interleukin-1 beta (M86725).

In a subsequent step, aligned sequences were examined to determine the codons corresponding to each aminoacid in the GP5 protein. For each nucleotide position the rate of mutation (percentage of strains having a different nucleotide) was calculated compared to LV. Also, for each codon the ratio between synonymous and non-synonymous mutations (S/NS) was determined. As an initial criterion, codons where at least 25% of the examined strains had a mutation and had an S/NS higher than 3 were considered as potentially negatively selected while ratios below 0.3 were potential points for positive selection. These thresholds were set arbitrarily. In the third step, the probability that n strains from a set of N sequences shared the same mutation was calculated. For this calculation it was assumed that each sequence was epidemiologically unrelated to the others. Considering the degeneracy of the genetic code, the probability of a synonymous mutation (Ps) for a given aminoacid at a given point was calculated as Ps = (codons coding the same aminoacid-1)/60. The probability of a non-synonymous mutation was 1-Ps. The probability that n unrelated sequences shared a mutated codon encoding the same aminoacid was calculated according to a binomial distribution. Positive or negative selection at a given point was arbitrarily considered to occur when the probability was $\leq 1 \times 10^{-9}$.

Predicted GP5 aminoacid sequences were aligned, similarities to LV were calculated and a bootstrapped phylogenetic tree was constructed using the neighbor-joining method (1000 iterations) using LV as the outgroup. An entropy plot of predicted GP5 was constructed to determine conserved and highly variable regions of the protein.

The sequence of a hypothetical strain containing all positively selected mutations was written and analysed using Bio-edit. The hypothetical GP5 was compared with other available European GP5 sequences using the Blastp utility (http://www.ncbi.nlm.nih.gov/BLAST). Finally, for this hypothetical strain transmembrane regions and N-glycosylation sites were evaluated using TMPred and NetNGlyc utilities at Expasy server (http://www.expasy.org) and the hydrophobic profile (Kyle and Dolittle method) was determined using Bio-edit.

For the Spanish 1991–1995 set of nucleotide sequences, the percentage of similarity to LV ranged from 99.1 to 87.2% (average, $95.5 \pm 3.6\%$). For the 2000–2005 set, similarity to LV ranged from 94.9 to 81.7% (average $89.5 \pm 2.8\%$). Entropy analysis showed three highly variable regions. The first one was located between nucleotide residues 165 and 189; the second one between residues 315 and 339 and the third one was located between residues 360 and 369. Other points of high variability were found at residues 36–39, 429–432 and 480–489. Regions located between nucleotides 99–120 and 123–165 were the less variable part.

As expected, the CA globally showed that codon usage was not significantly different in 1991–1995 and 2000–2005. However, when specific aminoacids were examined, codon usage differed among 1991–1995 sequences and those of 2000–2005 for leucine, glutamine, serine and proline (Table 1). Thus, predominant codon for leucine in the 1991–1995 set was CTC (RCSU = 2.08) while in 2000–2005 was TTG (RCSU = 1.99). For glutamine, the only codon present in the older set of

Table 1
Codon usage in the ORF5 gene of Spanish sequences of porcine reproductive and respiratory syndrome strains (1991–1995 and 2000–2005)

Cumulative Codon Usage (PRRSV isolates 2000-2005)								Cumulative Codon Usage (PRRSV isolates 1991-1995)							
AA	**Codon**	**N**	**RSCU**	**AA**	**Codon**	**N**	**RSCU**	**AA**	**Codon**	**N**	**RSCU**	**AA**	**Codon**	**N**	**RSCU**
Phe	**UUU**	439	**1,40**	Cys	UGU	186	0,98	Phe	**UUU**	180	**1,37**	Cys	UGU	58	0,89
	UUC	188	0,60		**UGC**	192	**1,02**		UUC	83	0,63		**UGC**	72	**1,11**
Leu	UUA	22	0,14	Ser	UCU	156	1,26	Leu	UUA	5	0,09	Ser	UCU	45	1,12
	UUG	322	**1,99**		**UCC**	181	**1,46**		UUG	120	2,05		UCC	63	1,56
	CUU	174	1,07		UCA	71	0,57		CUU	49	0,84		UCA	18	0,45
	CUC	284	1,75		UCG	108	0,87		**CUC**	122	**2,08**		UCG	24	0,60
	CUA	15	0,09		AGU	51	0,41		CUA	1	0,02		AGU	5	0,12
	CUG	155	0,96		AGC	177	1,43		CUG	55	0,94		**AGC**	87	**2,16**
Tyr	UAU	152	0,87	Trp	**UGG**	320	**1,00**	Tyr	UAU	51	0,78	Trp	**UGG**	110	**1,00**
	UAC	197	**1,13**						**UAC**	80	**1,22**				
His	**CAU**	205	**1,24**	Pro	CCU	39	0,64	His	**CAU**	87	**1,55**	Pro	CCU	10	0,54
	CAC	126	0,76		**CCC**	79	**1,30**		CAC	25	0,45		CCC	19	1,03
					CCA	65	1,07						CCA	19	1,03
Gln	**CAA**	152	**1,72**		CCG	61	1,00	Gln	**CAA**	39	**2,00**		**CCG**	26	**1,41**
	CAG	25	0,28						CAG	0	0,00				
Ile	AUU	95	0,69	Arg	CGU	116	1,35	Ile	AUU	31	0,64	Arg	CGU	38	1,35
	AUC	166	**1,20**		CGC	69	0,80		**AUC**	57	**1,18**		CGC	19	0,67
	AUA	154	1,11		CGA	49	0,57		**AUA**	57	**1,18**		CGA	16	0,57
					CGG	147	**1,71**						**CGG**	55	**1,95**
Met	**AUG**	107	**1,00**					Met	**AUG**	21	**1,00**				
				Thr	ACU	153	0,88					Thr	ACU	53	0,82
Asn	AAU	115	0,76		**ACC**	267	**1,53**	Asn	AAU	44	0,85		**ACC**	106	**1,64**
	AAC	187	**1,24**		ACA	172	0,99		**AAC**	60	**1,15**		ACA	63	0,97
					ACG	104	0,60						ACG	37	0,57
Lys	**AAA**	270	**1,57**					Lys	**AAA**	95	**1,68**				
	AAG	74	0,43	Arg	AGA	47	0,55		AAG	18	0,32	Arg	AGA	12	0,43
					AGG	89	**1,03**						**AGG**	29	**1,03**
Val	GUU	157	0,79					Val	GUU	59	0,75				
	GUC	299	**1,51**	Ala	**GCU**	313	**1,68**		**GUC**	119	**1,52**	Ala	**GCU**	126	**1,83**
	GUA	83	0,42		GCC	218	1,17		GUA	51	0,65		GCC	68	0,99
	GUG	254	1,28		GCA	115	0,62		GUG	85	1,08		GCA	47	0,68
					GCG	101	0,54						GCG	35	0,51
Asp	GAU	99	0,69					Asp	GAU	35	0,56				
	GAC	190	**1,31**	Gly	GGU	146	0,78		**GAC**	91	**1,44**	Gly	GGU	51	0,73
					GGC	318	**1,69**						**GGC**	137	**1,97**
Glu	GAA	156	0,94		GGA	78	0,41	Glu	**GAA**	52	**1,00**		GGA	18	0,26
	GAG	176	**1,06**		GGG	210	1,12		**GAG**	52	**1,00**		GGG	72	1,04

AA: aminoacid; *N*: number of effective codons; RSCU: synonymous codon usage measure. In bold is shown the most frequent codon for each aminoacid. Boxes show the main changes between 1991–1995 and 2000–2005 sequences.

sequences was CAA (RSCU = 2.0) while in the set 2000–2005 appeared the codon CAG (RSCU = 0.28). For serine, older strains preferentially carried AGC (RSCU = 2.16) while newer strains preferentially used TCC (RSCU = 1.46) and for proline, older strains preferentially had CCG (RSCU = 1.41) instead of the CCC codon of the newer strains (RSCU = 1.30). Results for 1991–1995 non-Spanish European-type sequences were similar. The most frequent codons used for leucine were TTG (RSCU = 2.09) and CTC (RSCU = 1.97); for glutamine CAA (RSCU = 1.81) and CAG (RSCU = 0.19), for serine AGC (RSCU = 1.87) and TCC (RSCU = 1.41); and for proline CCG (RSCU = 1.84) and CCA (RSCU = 1.05). Some differences related to the country of origin were observed. Polish strains and the British strain preferentially coded leucine with the codon CTC. The codification of glutamine with the codon CAG was only found in Belgian strains.

Ten highly expressed pig genes were also analysed for codon usage. In those genes, the most commons codons for leucine, glutamine, serine and proline were TTG (RSCU = 0.81); CAG (RSCU = 1.45); TCC (RSCU = 1.36) and CCC (RSCU = 1.26).

Table 2
Potential sites for positive selection of mutations in Spanish strains of porcine reproductive and respiratory syndrome virus GP5

Position in GP5	Lelystad virus	Spanish strains 2000–2005 ($n = 47$)[a]			Spanish strains 1991–1995 ($n = 17$)		
	Aminoacid	Aminoacid (strains)	NS/S[b]	Probability[a]	Aminoacid (strains)	NS/S[b]	Probability[a]
10	Phenilalanine	Serine (14)	18/3	1.3×10^{-20}	No change	0/0	N.A.
12	Threonine	Isoleucine (12)	20/0	1.8×10^{-13}	No change	0/0	N.A.
13	Proline	Glutamine (12)	21/18	1.3×10^{-18}	Leu (1)	2/8	0.03
		Leucine (7)		3.2×10^{-6}			
21	Phenilalanine	Serine (5)	6/0	7.5×10^{-9}	No change	0/0	N.A.
32	Alanine	Valine (15)	19/0	9.6×10^{-17}	Valine (5)	5/0	3.2×10^{-7}
36	Glycine	Aspartic acid (12)	17/2	2.1×10^{-17}	Aspartic (2)	3/0	8.0×10^{-3}
37	Aspartic acid	Asparagine (38)	0/47	1.5×10^{-53}	Asparagine (5)	9/0	1.5×10^{-7}
		Serine (5)		9.7×10^{-4}	Serine (4)		8.9×10^{-6}
46	Asparagine	Aspartic acid (12)	15/14	1.5×10^{-12}	No change	0/0	N.A.
56	Aspartic acid	Glycine (16)	42/0	6.7×10^{-11}	Glutamic acid (4)	7/1	2.6×10^{-6}
		Glutamic acid (14)		4.2×10^{-15}			
60	Serine	Asparagine (16)	33/0	6.2×10^{-33}	Asparagine (1)	2/0	0.03
63	Glycine	Aspartic acid (18)	45/0	1.1×10^{-20}	Aspartic acid (11)	12/0	3.2×10^{-19}
		Serine (12)		5.7×10^{-12}			
96	Glycine	Serine (13)	15/10	7.8×10^{-19}	Serine (2)	2/1	8.0×10^{-4}
100	Threonine	Isoleucine (11)	24/0	9.1×10^{-11}	Isoleucine (3)	5/0	3.4×10^{-4}
101	Alanine	Threonine (30)	46/0	1.1×10^{-29}	Threonine (8)	9/0	1.6×10^{-9}
106	Glycine	Lysine (16)	26/3	4.8×10^{-15}	Lysine (4)	3/1	3.8×10^{-7}
111	Cysteine	Serine (36)	47/0	1.4×10^{-54}	Serine (15)	15/0	2.1×10^{-27}
116	Alanine	Valine (17)	26/1	1.5×10^{-18}	Phenilalanine (1)	1/0	0.02
119	Phenilalanine	Leucine (9)	17/10	7.0×10^{-12}	Leucine (3)	3/1	1.3×10^{-6}
122	Phenilalanine	Leucine (30)	35/0	9.3×10^{-42}	Leucine (9)	9/0	9.9×10^{-17}
123	Valine	Alanine (13)	19/11	8.6×10^{-12}	No change	0/0	N.A.
143	Phenilalanine	Histidine (9)	12/9	4.1×10^{-14}	No change	0/0	N.A.
154	Valine	Isoleucine (39)	42/0	4.9×10^{-66}	Isoleucine (10)	10/0	1.7×10^{-15}
172	Aspartic acid	Glycine (34)	41/5	2.6×10^{-39}	Glycine (10)	10/6	5.7×10^{-10}
174	Asparagine	Aspartic acid (35)	43/0	1.7×10^{-52}	Aspartic acid (10)	11/0	1.8×10^{-17}

[a] Probability: probability, according to a binomial distribution, that in a set of n independent sequences with a mutation, a given number of strains shared the same non-synonymous mutation. For strains detected in the period 2000–2005 only mutations with probabilities lower than 1.0×10^{-9} are shown.
[b] NS/S: non synonymous/synonymous mutations.

The analysis of codons present in each sequence showed an overall S/NS of 1.41. In the set of sequences from 2000 to 2005, 24 codons showed the characteristics of a positive selection according to the calculated probabilities (Table 2). Interestingly, 12 strains changed Asn-46 to Asp-46, losing thus one glycosylation site. Also, 11 of those 12 strains gained a glycosylation site by changing Asp-37 to Asn-37. For the 1991–1995 set, the number of positively selected sites was seven. Another 20 codons showed the characteristics of a negative selection and were mainly distributed in three segments of the predicted protein (residues 73–89; 108–113 and 153–169) (Table 3). Codon usage significantly changed ($p < 0.05$) in 14 of these negatively selected positions between strains of 1991–1995 and those of 2000–2005 (Table 4).

Average similarity of predicted GP5 proteins with regards to LV was 83.8% for the 2000–2005 set and 94.4% in the 1991–1995 set. The bootstrapped tree of the predicted aminoacid sequences of GP5 a high diversity in GP5 (Fig. 1). Although, in general, strains from 2000 to 2005 tended to cluster together while 1991–1995 sequences were scattered along the tree, bootstrap values only supported small clusters and did not provide evidence for a clear evolutionary line between modern and older strains.

The entropy analysis showed three highly variable regions located between aminoacids 56–63; 105–113 and 120–130. These segments corresponded to the parts with higher entropy values for the nucleotide sequence. The most conserved region was found between residues 38–55.

A hypothetical GP5 containing all positively and negatively selected sites was analysed to make a prediction of its characteristics. This hypothetical GP5 had a similarity of 88.1% compared to LV. As shown by BLAST comparison, the 10 sequences closest to the hypothetical GP5 (besides those included in the study) had a similarity ranging from 91 to 88% (average 89.4%). The older strain included in this set of 10 was a Spanish sequence of 1991. Interestingly, of the 24 predicted positive selection sites, 16 were present in sequences from other European countries and eight were predominant (>70% frequency) regardless of the country of origin of the PRRSV strain. Regarding negative selection sites, 19 out of 20 were present in all sequences (Fig. 2).

For the hypothetical strain, the signal peptide comprised residues 1–34 (1–32 in LV); transmembrane regions were pre-

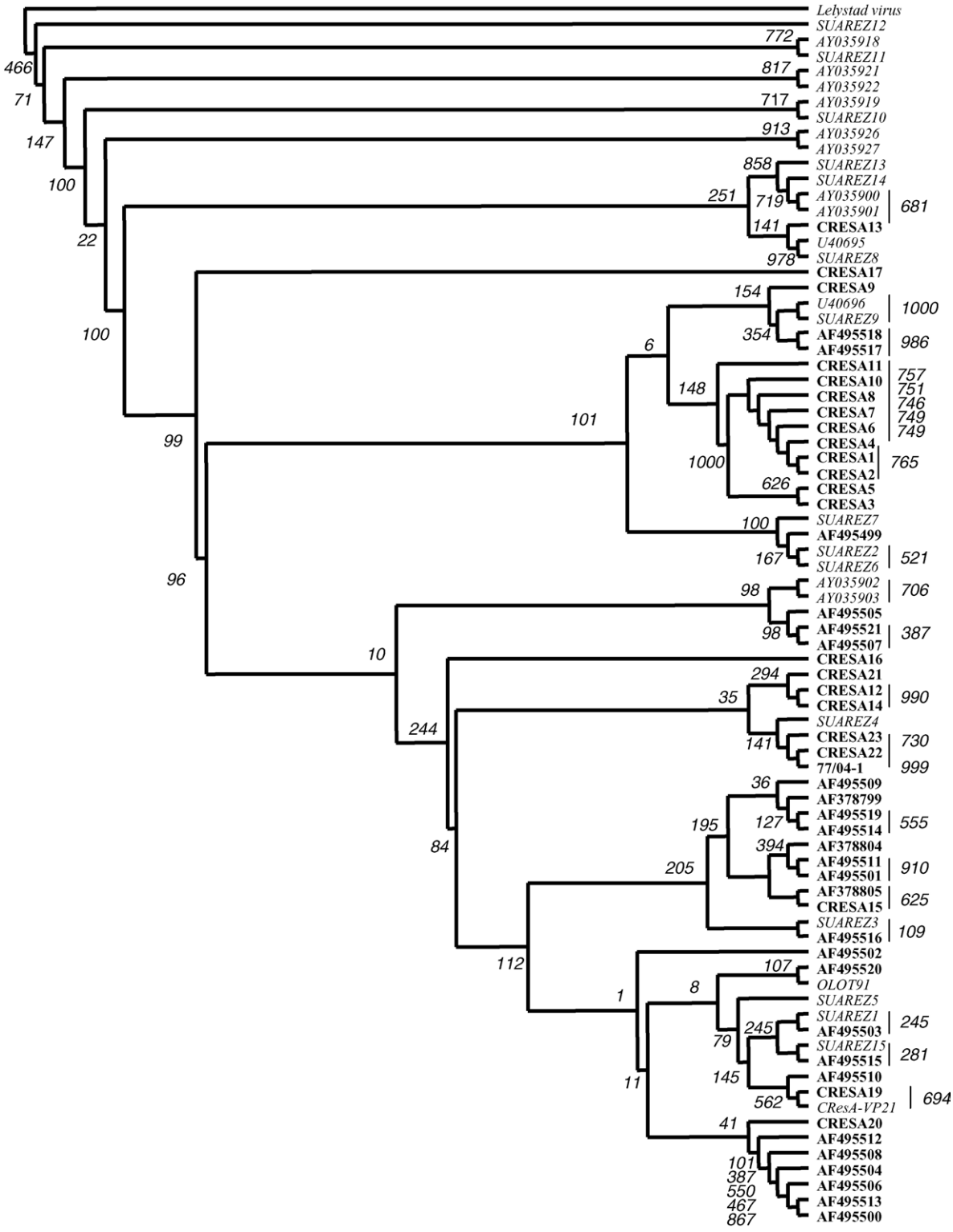

Fig. 1. Bootstrapped tree (neighbor-joining method) of predicted aminoacid sequences of GP5 of PRRS virus. In italics (light colour) sequences from 1991 to 1995 period; in bold type, sequences from 2000 to 2005. Spanish strains 1991–1995 = Suarez, CRESA-VP21 and Olot91. Spanish Strains 2000–2005 = AF49XXX and CReSA. All other strains correspond to European non-Spanish isolates from 1991 to 1995. Bootstrap values are shown in italics close to the nodes.

dicted to exist at the following segments: 69–90 and 108–127 (same segments in LV). Potential *N*-glycosylation sites were predicted at residues 37 and 53 (46 and 53 in LV). Comparison of the hydrophobicity profiles of LV and the hypothetical GP5 showed that the latter was less hydrophobic (not shown).

The study of the evolution and adaptation of viruses to their hosts is a question of relevance because provides insight on the

Table 3
Potential sites for negative selection of mutations in Spanish strains of porcine reproductive and respiratory syndrome virus GP5 obtained in 2000–2005

Position in GP5	Aminoacid	S/NS[a]	Probability[b]
8	Glycine	20/2	2.0×10^{-24}
73	Proline	17/1	1.3×10^{-21}
79	Leucine	33/4	1.3×10^{-54}
85	Threonine	15/4	9.6×10^{-17}
89	Phenilalanine	16/3	3.3×10^{-26}
91	Aspartic acid	31/4	3.69×10^{-51}
108	Tyrosine	34/8	3.6×10^{-53}
109	Valine	21/7	3.9×10^{-22}
112	Serine	22/8	3.9×10^{-33}
113	Valine	34/8	4.6×10^{-37}
135	Alanine	36/5	8.4×10^{-42}
137	Arginine	28/5	9.3×10^{-26}
140	Arginine	25/7	1.9×10^{-21}
153	Arginine	35/5	2.5×10^{-31}
160	Proline	16/3	1.2×10^{-18}
163	Valine	25/4	5.8×10^{-29}
164	Glutamic acid	39/3	4.9×10^{-66}
169	Alanine	21/7	3.9×10^{-22}
194	Threonine	14/4	1.5×10^{-15}
200	Glutamic acid	35/8	7.4×10^{-55}

[a] S/NS: synonymous/non-synonymous mutations.
[b] Probability: probability, according to a binomial distribution, that in a set of n independent sequences with a mutation, a given number of strains shared a synonymous mutation.

mechanisms by which a viral variant gains prevalence in a population. A large endemic population with a high replacement rate is a suitable frame to study such phenomena. This is the case of Spain for PRRSV. Spanish pig population is the second largest in Europe with some 24 million pigs and, according to FAO statistics, imports every year about 1.2 million live pigs (http://faostat.fao.org).

The present study was conducted with two sets of PRRSV sequences, one corresponding to the period 1991–1995 and the other to 2000–2005. In this lapse of years, average similarity to LV changed from above 95% in 1991–1995 to below 90% in 2000–2005. These values suggest an increase in divergence of about 0.5% per year. If divergence increased at a constant rate and sequences from 1991 to 1995 shared an average similarity of 95% to LV, original PRRSV strains in pigs could have originated some 10 years before; namely, about 1981–1985. This is the predicted date in which PRRSV is thought to have entered the domestic pig population (Forsberg et al., 2001; Hanada et al., 2005; Plagemann, 2003).

The entropy analysis showed that this divergence arise from mutations scattered in ORF5 although hypervariable regions could be recognised. This has been described before (Pesch et al., 2005; Pirzadeh et al., 1998) and it is thought that these hypervariable regions can correspond to potentially immunogenic sites. Actually, the neutralisation epitope of GP5 is located in the middle of the ectodomain (Plagemann, 2004b) and the first hypervariable region flanked this epitope.

Codon usage was different for leucine, glutamine, serine and proline in either set of Spanish sequences. When compared with the codon usage of other early European PRRSV strains, results were similar for glutamine, serine and proline while

Table 4
Significant changes ($p < 0.05$) in codon usage for negatively selected sites of ORF5 of porcine reproductive and respiratory strains isolated in 1991–1995 or 2000–2005

Position in GP5	Aminoacids	Codons 1991–1995[a]	Codons 2000–2005[a]
73	Proline	CCG (18/18)	CCG (30/46) CCA (15/46) CCC (1/46)
79	Leucine	CTC (12/18) CTT (6/18)	CTC (11/44) CTT (33/44)
85	Threonine	ACA (18/18)	ACA (29/45) ACG (13/45) ACC (3/45)
89	Phenilalanine	TTT (18/18)	TTT (29/46) TTC (17/46)
91	Aspartic acid	GAC (11/18) GAT (7/18)	GAC (13/45) GAT (32/45)
109	Valine	GTA (15/17) GTG (2/17)	GTA (20/41) GTG (21/41)
112	Serine	AGC (15/18) AGT (3/18)	AGC (18/40) AGT (22/40)
135	Alanine	GCC (7/17) GCT (10/17)	GCC (7/43) GCT (34/43) GCG (2/43)
140	Arginine	CGT (13/17) CGC (4/17)	CGC (16/40) CGC (24/40)
153	Arginine	AGA (8/18) AGG (10/18)	AGA (8/43) AGG (35/43)
160	Proline	CCA (18/18)	CCA (29/45) CCG (11/45) CCC (5/45)
163	Valine	GTA (13/15) GTG (2/15)	GTA (18/43) GTG (16/43) GTC (9/43)
164	Glutamic acid	GAA (16/18) GAG (2/18)	GAA (6/45) GAG (39/45)
169	Alanine	GCC (10/18) GCT (8/18)	GCC (20/41) GCT (21/41)

[a] Differences in the denominator to 18 (1991–1995 sequences) or to 46 (2000–2005 strains) reflect non-synonymous mutations.

leucine was preferentially coded with TTG as did the most recent Spanish strains. Most frequent codons for these aminoacids in 2000–2005 sequences were similar to the codon usage in some highly expressed swine genes. These results can be interpreted as a sign of either a selection or an adaptation of PRRSV to the codon usage most adequate for an efficient replication in the pig host. Also, this adaptation can have other implications. Several authors (Cook et al., 2005; Kheyar et al., 2005) have shown that optimising codon usage of arterivirus genes to that of mammalian cells results in an increase of the levels of expression of viral genes as well as increases immunogenicity of viral proteins. In the present case, our results suggest that PRRSV is still adapting to the swine host. This should be taken into account when designing attenuated vaccines because adequate

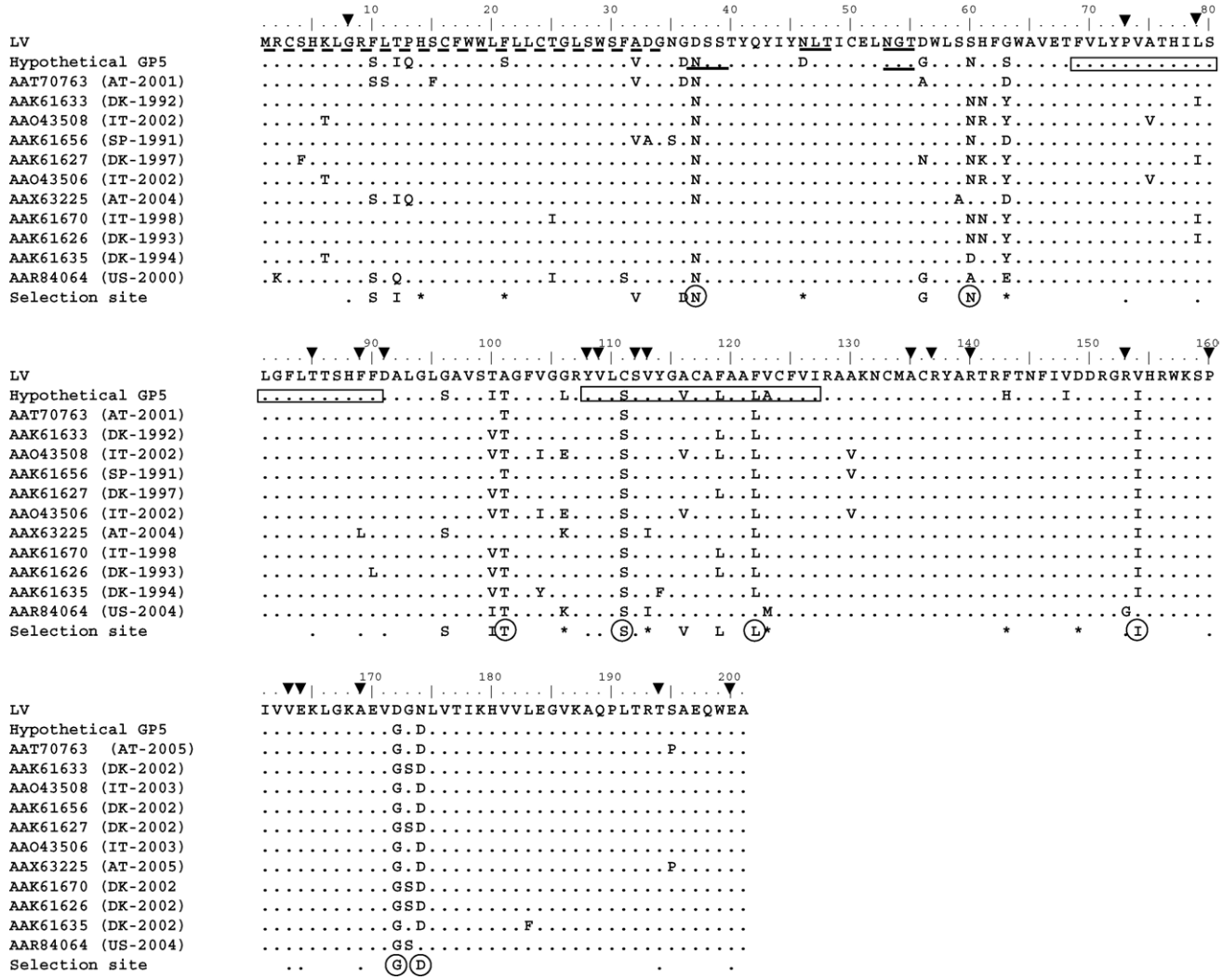

Fig. 2. Comparison with other non-Spanish European strains of the hypothetical GP5 containing all predicted sites for positive and negative selection in Spanish PRRSV sequences. The hypothetical GP5 was BLASTed and the 10 closest non-Spanish matches were included in the alignment along with Lelystad virus GP5 (LV) and an European-type strain isolated in USA. Protein sequences are represented by its GenBank accession number followed by the country of origin (AT: Austria, DK: Denmark, IT: Italy, US: United States of America) and the year of isolation. Amino acids are presented with a one-letter code in the row corresponding to LV. (- - -) Discontinuous underlined: predicted signal peptide. (—) Continuous underlined: predicted *N*-glycosylation sites. (▭) Boxed segments: predicted transmembrane regions in the hypothetical GP5. (▼) Negative selection sites. Positive selection sites are marked with the one-letter aminoacid code in the hypothetical GP5. Selection site: the last row shows whether or not the predicted selection sites were found in other non-Spanish European sequences. Dots (·) indicate negative selection site found in 11/11 non-Spanish European sequences. Aminoacid symbol (X) indicates positive selection site found in at least one non-Spanish European sequence. Encircled aminoacid symbol (®) indicates positive selection site found in ≥8/11 non-Spanish European sequences. (*) Predicted positive or negative selection site not found in non-Spanish European sequences.

levels of expression of viral proteins are important to develop strong immune responses.

The codon analysis revealed and average S/NS of 1.41. This ratio was similar to that determined by Hanada et al. (2005) for Coronaviruses but lower than reported by others (Pesch et al., 2005) for PRRSV. This high rate of non-synonymous mutations may have important implications for the design of vaccines since these variable points may constitute inefficient targets for the immune system.

The examination of positively and negatively selected sites showed 24 potential sites for positive selection and 20 for negative selection. Ten of the 24 sites for positive selection were located in transmembrane sections of the predicted GP5, a fact that suggests that many of these adaptations were not selected because of a pressure of the neutralising antibodies. In contrast, the negatively selected sites were concentrated in the last 100 aminoacids of GP5 (14/20 sites) for which no neutralising antibodies have been detected so far (Plagemann, 2004a,b). These negatively selected sites clustered in three segments of the predicted GP5. The first two of these clusters (residues 73–89 and 108–113) corresponded to predicted transmembrane regions while the function of the third segment is still unknown. Since variability of aminoacids in those points is restricted in spite of the presence of several possible codons, it is reasonable to think that these sites are probably crucial for virus integrity or functionality.

The phylogenetic analysis of GP5 sequences did not support a clear line of evolution from older to newer Spanish strains. However, clustering was evident for some newer sequences. With the available data it is impossible to know whether the newer strains represent a PRRSV type that slowly evolved from older strains or if they represent an old type that gained predominance. Unfortunately, we were not able to obtain Spanish sequences from 1996 to 1999 and therefore we could not fill this gap; however, the analysis of 153 GP5 European-type sequences from different countries and periods yielded similar results (not shown). The closest matches to the hypothetical GP5 included strains from different countries and periods but the oldest known match was a Spanish strain from 1991 not included in this study. This fact would support the hypothesis that similarity to the hypothetical GP5 has some benefit because this profile has become predominant in Spain all over the years.

The hypothetical strain was similar to LV but had two additional characteristics that lacked in LV. The first was a change in the sites of glycosylation compared to LV. One was located at the start of the ectodomain (Asp-37) and the second was located at the known neutralisation epitope (Asp-53). This hypothetical GP5 would lose thus the glycosylation site at Asp-46. In contrast, positive selection in position 37 introduces a new glycosylation site. Previous studies suggested that the lack of this glycosylation at Asp-46 reduces virus infectivity and can be a marker of attenuation (Wissink et al., 2004). However, according to Pesch et al. (2005), glycosylation at position 46 can be found in three European-type attenuated vaccines of which only one also has a glycosylation at position 37. Several authors claimed that these additional glycosylation sites may serve to mask the key B-epitopes (Chen et al., 1998) although this has not been proven yet for PRRSV. The second fact that differentiates the hypothetical GP5 from that of LV was a lesser hydrophobic profile. The consequence would be a more exposed GP5 that could better interact with the receptors in target cells.

The present study shows that ORF5 of PRRSV has increased its genetic diversity over time. This evolution included positive and negative selections of given aminoacids in specific sites of the PRRSV genome, mainly in transmembrane segments of GP5. Also, codon usage for leucine, glutamine, serine and proline changed and in more recent sequences have more resemblance to codon usage in highly expressed pig genes suggesting that a process of adaptation to pig is taking place. These data, if further confirmed by other studies with PRRSV isolates of other countries, may be useful to understand the evolution of PRRSV as well as can be relevant for the design of new and more efficacious vaccines.

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References

Castro, C., Arnold, J.J., Cameron, C.E., 2005. Incorporation fidelity of the viral RNA-dependent RNA polymerase: a kinetic, thermodynamic and structural perspective. Virus Res. 107, 141–149.

Chen, Z., Li, K., Rowland, R.R., Plagemann, P.G., 1998. Neuropathogenicity and susceptibility to immune response are interdependent properties of lactate dehydrogenase-elevating virus (LDV) and correlate with the number of N-linked polylactosaminoglycan chains on the ectodomain of the primary envelope glycoprotein. Adv. Exp. Med. Biol. 440, 583–592.

Cook, R.F., Cook, S.J., Bolin, P.S., Howe, L.J., Zhou, W., Montelaro, R.C., Issel, C.J., 2005. Genetic immunization with codon-optimized equine infectious anemia virus (EIAV) surface unit (SU) envelope protein gene sequences stimulates immune responses in ponies. Vet. Microbiol. 108, 23–37.

Fang, Y., Kim, D.Y., Ropp, S., Steen, P., Christopher-Hennings, J., Nelson, E.A., Rowland, R.R., 2004. Heterogeneity in Nsp2 of European-like porcine reproductive and respiratory syndrome viruses isolated in the United States. Virus Res. 100, 229–235.

Forsberg, R., Oleksiewicz, M.B., Petersen, A.M., Hein, J., Botner, A., Storgaard, T., 2001. A molecular clock dates the common ancestor of European-type porcine reproductive and respiratory syndrome virus at more than 10 years before the emergence of disease. Virology 289, 174–179.

Forsberg, R., Storgaard, T., Nielsen, H.S., Oleksiewicz, M.B., Cordioli, P., Sala, G., Hein, J., Botner, A., 2002. The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. Virology 299, 38–47.

Gonin, P., Pirzadeh, B., Gagnon, C.A., Dea, S., 1999. Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP5 major envelope glycoprotein. J. Vet. Diagn. Invest. 11, 20–26.

Hanada, K., Suzuki, Y., Nakane, T., Hirose, O., Gojobori, T., 2005. The origin and evolution of porcine reproductive and respiratory syndrome viruses. Mol. Biol. E 22, 1024–1031.

Kheyar, A., Jabrane, A., Zhu, C., Cleroux, P., Massie, B., Dea, S., Gagnon, C.A., 2005. Alternative codon usage of PRRS virus ORF5 gene increases eucaryotic expression of GP(5) glycoprotein and improves immune response in challenged pigs. Vaccine 23, 4016–4022.

Mateu, E., Martin, M., Vidal, D., 2003. Genetic diversity and phylogenetic analysis of glycoprotein 5 of European-type porcine reproductive and respiratory virus strains in Spain. J. Gen. Virol. 84, 529–534.

Meng, X.J., Paul, P.S., Halbur, P.G., 1994. Molecular cloning and nucleotide sequencing of the 3′-terminal genomic RNA of the porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 75, 1795–1801.

Oleksiewicz, M.B., Botner, A., Toft, P., Grubbe, T., Nielsen, J., Kamstrup, S., Storgaard, T., 2000. Emergence of porcine reproductive and respiratory syndrome virus deletion mutants: correlation with the porcine antibody response to a hypervariable site in the ORF 3 structural glycoprotein. Virology 267, 135–140.

Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A., Lopez, O.J., 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J. Virol. 76, 4241–4250.

Pesch, S., Meyer, C., Ohlinger, V.F., 2005. New insights into the genetic diversity of European porcine reproductive and respiratory syndrome virus (PRRSV). Vet. Microbiol. 107, 31–48.

Pirzadeh, B., Gagnon, C.A., Dea, S., 1998. Genomic and antigenic variations of porcine reproductive and respiratory syndrome virus major envelope GP5 glycoprotein. Can. J. Vet. Res. 62, 170–177.

Plagemann, P.G., 2003. Porcine reproductive and respiratory virus: origin hypothesis. Emerg. Infect. Dis. 9, 903–905.

Plagemann, P.G., 2004a. GP5 ectodomain epitope of porcine reproductive and respiratory syndrome virus, strain Lelystad virus. Virus Res. 102, 225–230.

Plagemann, P.G., 2004b. The primary GP5 neutralization epitope of North American isolates of porcine reproductive and respiratory syndrome virus. Vet. Immunol. Immunopathol. 102, 263–275.

Suárez, P., Zardoya, R., Martin, M.J., Prieto, C., Dopazo, J., Solana, A., Castro, J.M., 1996. Phylogenetic relationships of european strains of porcine reproductive and respiratory syndrome virus (PRRSV) inferred from DNA sequences of putative ORF-5 and ORF-7 genes. Virus Res. 42, 159–165.

Snijder, E.J., Dobbe, J.C., Spaan, W.J., 2003. Heterodimerization of the two major envelope proteins is essential for arterivirus infectivity. J. Virol. 77, 97–104.

Snijder, E.J., Meulenberg, J.J., 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79, 961–979.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Wissink, E.H., Kroese, M.V., Maneschijn-Bonsing, J.G., Meulenberg, J.J., van Rijn, P.A., Rijsewijk, F.A., Rottier, P.J., 2004. Significance of the oligosaccharides of the porcine reproductive and respiratory syndrome virus glycoproteins GP2a and GP5 for infectious virus production. J. Gen. Virol. 85, 3715–3723.

Wu, W.H., Fang. Y., Rowland. R.R., Lawson, S.R., Christopher-Hennings, J., Yoon, K.J., Nelson, E.A., 2005. The 2b protein as a minor structural component of PRRSV. Virus Res [Epub ahead of print August 10].

7.2 Aportaciones a congresos internacionales

Use of ELISPOT and ELISA to evaluate g-IFN, IL-4 and IL-10 responses in pigs

Proceedings of the 18th International Pig Veterinary Society Congress, Hamburg, Germany, 2004, Volume I, p.425

Evolution of immunological parameters in experimental infection with an european-type PRRS virus

Proceedings of the 18th International Pig Veterinary Society Congress, Hamburg, Germany, 2004, Volume I, p.126

Immune response of pigs after a DNA vaccination with prrs virus ORF 7

Proceedings of the 19th International Pig Veterinary Society Congress, Copenhagen, Denmark, 2006, abstract 807

Evolution of the phagocytic capacity of neutrophils and macrophages in weaned pigs

Proceedings of the 19th International Pig Veterinary Society Congress, Copenhagen, Denmark, 2006, abstract 812

USE OF ELISPOT AND ELISA TO EVALUATE g-IFN, IL-4 AND IL-10 RESPONSES IN PIGS

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Introduction and Objectives

ELISPOT is a powerful technique for quantitation of cytokine responses, but its use has been scarce in veterinary immunology. In the case of swine, ELISPOT has been developed for the quantitation of gamma-IFN and alfa-IFN responses (1, 2) but, up to date, no other cytokines are known to be evaluated by this technique. The aim of this study was to standardize the ELISPOT technique for evaluating gamma-IFN, IL-10 and IL-4 in pigs from birth to six months of age, and to correlate the frequencies of cytokine-secreting cells (CSC) with the amount secreted cytokines as determined by ELISA.

Materials and Methods

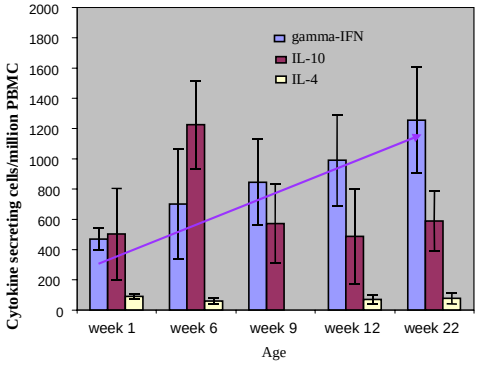
Five one-week old conventional piglets were randomly selected in a conventional farm and were bled at 1, 6, 9, 12 and 22 weeks of age. ELISA plates were coated overnight with gamma-IFN, IL-10 or IL-4 capture antibodies (Swine gamma-IFN, IL-10 and IL-4 Cytosets™, Biosource Europe). Peripheral blood mononuclear cells (PBMC) were dispensed in coated plates at different densities ranging from $2{,}5x10^4$ cells/well to $2x10^5$ cells/well. Cultures were stimulated with PHA or were mock-stimulated. After 20 h of incubation at 37ºC in 5 % CO_2 atmosphere, cells were removed and supernatants were stored at -80ºC until further examination. Then, the appropriate biotinilated detection antibody was added. Reaction was revealed by sequential incubation of plates with streptavidin-peroxidase and insoluble TMB (Calbiochem). Frequencies of cytokine producing cells were expressed as the number of responding cells per 10^6 PBMC. For this count, the number of spots in non-stimulated cultures was subtracted from the count of PHA wells. Cytokine levels in cell culture supernatants were evaluated in ELISA using the same antibody pairs than in ELISPOT. Cytokine concentrations were calculated from a regression equation obtained from optical density results of gamma-IFN, IL-10 and IL-4 standards provided with the antibody pairs. Statistical analysis of the evolution of CSC over time was done by means on the linearity test. Comparisons between the amounts of secreted cytokines with the number of CSC were done by regression analysis using Statsdirect®.

Results and discussion

Results are summarized in Figure 1. Frequencies of gamma-IFN-secreting cells (g-IFNSC) and IL-10-secreting cells (10SC) had a great interindividual variability. For gamma-IFN, the frequency of CSC increased with age ($p<0.05$) from an average of $486/10^6$PBMC at one week age to $1256/10^6$ PBMC at 22 weeks of age. In contrast, frequencies of 10SC were constant all over time ($537\pm40/10^6$ PBMC), except at six weeks of age where an increase was noticed ($1224\pm292/10^6$ PBMC; $p<0.05$). Spontaneous secretion of IL-10 was observed in all animals at all ages. Regarding IL-4, the frequencies of CSC were constant ($70\pm20/10^6$ PBMC). When cytokine levels where determined by ELISA no correlation was found with CSC levels, except for IL-10 ($r=0{,}74$; $p<0.05$). IL-4 was scarcely detected in ELISA in spite that CSC were found in ELISPOT.

Our results show that, under natural conditions, frequencies of g-IFNSC increase with age. This could be probably attributable to the increase of double positive CD4/CD8 lymphocytes which are known to be memory/effector cells with a great ability to secrete gamma-IFN (3). The constant frequencies of IL-4 and IL-10 are difficult to explain but suggest that they are not affected by changes in the proportion of memory cells. The lack of correlation between CSC frequencies and the levels of secreted cytokines indicated either a greater sensitivity of ELISPOT compared to ELISA (4) or that among CSC exist different subpopulations of high and low secretion abilities that can vary overtime.

Figure 1



References

(1) Charley B. *et al*. 1995. Vet Res 26:4 292-299.
(2) Splichal I. *et al.* 1997. Immunol Lett 43 :3 203-208.
(3) Zuckermann F.A. 1999. Vet Imml Immunopath 72: 55-66.
(4) Tanguay S and Killion JJ. 1994. Lymph Cyt Res. 13(4):259-63.

EVOLUTION OF IMMUNOLOGICAL PARAMETERS IN EXPERIMENTAL INFECTION WITH AN EUROPEAN-TYPE PRRS VIRUS

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Introduction and Objectives

Porcine reproductive and respiratory syndrome (PRRS) still is one of the major economic threats for the pig industry. In spite of the intensive research carried out since the discovery of the disease, many immunologic aspects of this infection are unknown. The development of effective vaccines is conditioned by this lack of knowledge. We present a preliminary characterization of cellular and humoral responses in pigs experimentally infected with an European-type strain of PRRS virus.

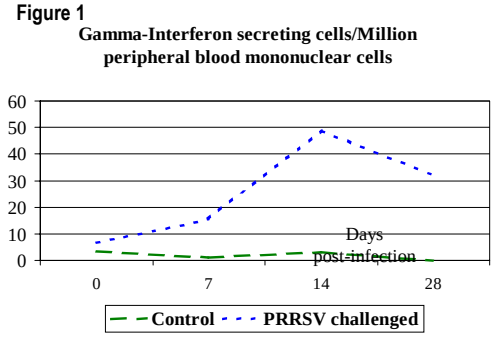
Materials and Methods

Ten twelve-weeks old healthy Landrace pigs were selected for this study. Animals were randomly distributed in two groups and were kept untreated during three weeks for acclimatization purposes. At 15 weeks of age were intranasally challenged with a Spanish isolate (VP-21) of PRRS virus. Body temperature was taken from day 0 to day 12 post-infection. Blood samples were collected at weekly intervals and peripheral blood mononuclear cells were obtained to determine in ELISPOT the frequencies of gamma-interferon (G-IFN) and IL-10 secreting cells and to evaluate by flow cytometry the changes of relative proportions of several cell subsets. Sera were analysed by immunoperoxidase monolayer assay (IPMA) to evaluate antibody responses. Weekly weight gains were recorded as an index of productive performance.

Results and Discussion

In infected animals, virus-specific G-IFN secreting cells appeared at day 7 post-infection (PI), reaching a peak on day 14 and then slowly declining until day 28 when its average frequency was 32 per million (Figure 1).

Figure 1



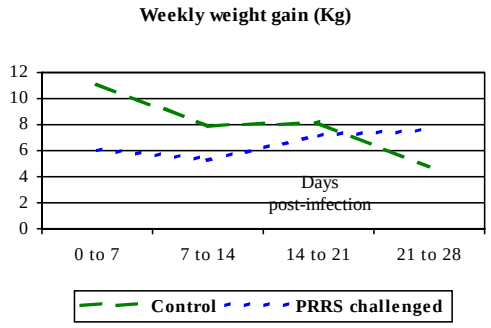
Regarding IL-10 secreting cells, no differences were seen among groups. Interestingly, spontaneous secretion of this cytokine was observed in most pigs.

Flow cytometric examination of blood subsets showed a transitory increase of CD8+ cells at day 7 PI ($p<0.05$) corresponding with the initial increase in G-IFN secreting cells. No other significant changes were observed for B cells (CD21), monocytes (SWC3) or activated lymphocytes (CD25).

In infected pigs, production of PRRS-specific antibodies was detected from day 7 PI onwards. At day 28, infected animals had IPMA titers from 1:640 to 1:10,240.

Clinical examinations showed that infected animals had a biphasic body temperature pattern. The first febrile phase lasted from day 2 PI to day 4 PI while the second started at day 10 PI and lasted until day 12 PI. With regards to the weekly weight gain, infected pigs grow was slower than that of controls from day 0 to 21 PI, showing afterwards a compensatory gain (Figure 2).

Figure 2



These results suggest that development of virus-specific G-IFN secreting cells correspond with a CD8+ increase and, in contrast with previous reports (1), in this case, IL-10 did not seem to play a major role in early phases of PRRS virus infection. The increase of G-IFN secreting cells corresponded to increases of CD8+ cells.

References

1) Suradhat et al. 2003. J Gen Virol, 84: 2755-2760.

IMMUNE RESPONSE OF PIGS AFTER A DNA VACCINATION WITH PRRS VIRUS ORF 7

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Introduction and Objectives

Currently, commercial vaccines for porcine reproductive and respiratory syndrome virus (PRRSV) are only partially effective when examined under an immunological point of view (1). Therefore, alternative vaccines are being investigated, among them, DNA vaccines (2). In this report, we present the preliminary results of an experimental DNA vaccine made of a plasmid that codifies PRRSV ORF7 and a chemokine.

Materials and Methods

DNA vaccines. PRRSV ORF7 (strain 5710) was obtained by RT-PCR from mRNA of infected MARC-145 cells. cDNA was cloned into plasmid pcDNA3.1. The complete coding sequence of porcine SLC was amplified by RT-PCR from RNA of alveolar macrophages and cloned into pcDNA 3.1 plasmid.

Animals and experimental design. Fourteen high health 4 weeks-old Landrace pigs were randomly distributed in four groups, namely A, B, C and D (table 1) and were allowed to acclimatise for two weeks. Then, at six weeks of age, animals were intramuscularly inoculated with either the plasmid encoding ORF7 (500 µg in 1ml), the ORF7 plasmid plus the SLC gene (500 µg in 1ml) or PBS (1ml). Inoculations were repeated every two times more three weeks apart each. At 14 weeks of age, groups B, C and D were intranasally challenged with a Spanish isolate (VP21) of PRRSV used in other studies (3).

Table 1

Group	Nº pigs	Treatment	Challenge
A	2	PBS	NO
B	4	PBS	YES
C	4	ORF 7	YES
D	4	ORF 7 + SLC	YES

Samples and Assays. Blood samples were collected at 0, 7, 14 and 21 days post-infection (PI). Sera were analyzed by the immunoperoxidase monolayer assay (IPMA) to evaluate antibody responses. Peripheral blood mononuclear cells (PBMC) were obtained to determine the frequencies of PRRS virus-specific IFN-γ-secreting-cells (IFNγ-SC) by ELISPOT (4) and to evaluate, by flow cytometry, the relative proportions of lymphocyte subsets. Weekly weight gains were recorded as an index of productive performance.

Results

Before the challenge, vaccinated pigs showed IPMA titers around 1:16. Antibodies titres clearly rose by day 7 PI in both vaccinated groups (?1:4,000 IPMA) (Figure 1). In group B, antibodies only were detected from 14 PI (1:400). Vaccinated pigs showed a significant increase of IFNγ-SC at 7 PI, being the mean of these groups from 4 to 10 times higher than in group B (Figure 2). SLC adjuvantation increased the IFNγ-SC response in some pigs, although this was not consistently seen.

Figure 1. Humoral response

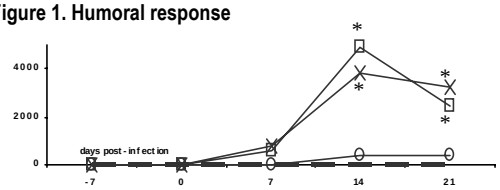
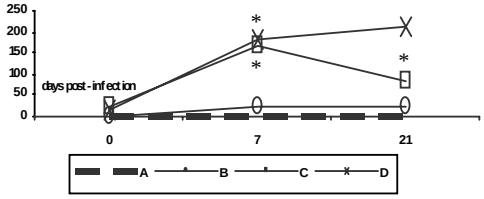


Figure 2. IFNγ-SC/Million PBMC



(*) P<0.05 compared to group B

All infected pigs showed a transitory increase of CD8+ lymphocytes and a transitory decrease of CD25+ and CD21+. The challenge produced a significant reduction in weight gain in groups B, C and D ($p<0.05$). However, while in group B (unvaccinated pigs) this decrease was noticeable from day 7 PI onwards, in vaccinated pigs growth retardation was only noticed by day 21 PI.

Discussion

The present study confirms that N protein of PRRSV is able to induce both humoral and cellular responses (5). However, the increase of IFNγ-SC in vaccinated pigs after the challenge could only be partially related to the protection against PRRSV as suggested by the different kinetics of growth retardation in vaccinated and unvaccinated pigs. The increase of $CD8^+$ and decrease of $CD25^+$ in infected pigs may suggest that PRRSV produced a polyclonal stimulation of lymphocytes that did not seem to be related to specific response (3). The decrease in CD21+ cells could be attributable to the mobilization of B cells to tissues, as previously suggested (3, 6). Finally, the SLC chemokyne might improve the immunogenicity of DNA vaccines against PRRSV, although this effect may depend on individual basis.

References

1. Meier et al (2003). Virology 309, 18-31.
2. Kwang et al (1999). Res Vet Sci 67, 199-201.
3. Díaz et al (2005). J Gen Virol 86, 1943-1951.
4. Díaz & Mateu (2005). Vet Imm Immunop 106, 107-112.
5. Rompato et al (2006) Vet Imm Immunop 109, 151-16.
6. Kawashima et al (1999) Vet Imm Immunop 71,257-262.

EVOLUTION OF THE PHAGOCYTIC CAPACITY OF NEUTROPHILS AND MACROPHAGES IN WEANED PIGS

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Introduction and Objectives

The role of neutrophils and macrophages is critical in the innate immune response. The aim of the present study was to determine the magnitude and the variation of the phagocytic capacity of macrophages and neutrophils in recently weaned pigs. Also the unspecific response of T cells was analyzed after stimulation with a mitogen.

Materials and Methods

Eight high health three weeks-old Landrace pigs were selected for this study. At 3, 4 and 8 weeks of age blood samples were collected to determine the number of neutrophils and macrophages and their phagocytic capacity. Phagocytic capacity of blood leukocytes was determined by means of a flow cytometry technique using *Streptococcus suis* type II cells labelled with FITC. The enriched fraction of phagocytic cells was purified by the dextran method. Phagocytic cells enriched fraction was incubated at 37ºC for 45 minutes with FITC labelled *S. suis.* After several washes, cells were counterstained with etidium bromide quench fluorescence of non-phagocyted bacteria, and then the cellular suspension was analyzed by flow cytometry.

A phagocytic index (PhI) was calculated as the average fluorescence channel multiplied by the percentage of cells with a fluorescence intensity above the background. In order to ascertain whether the observed changes in the PhI were attributable or not to the variation of each type of phagocytes, results were normalised by dividing the PhI by the percentage of neutrophils or macrophages that effectively phagocited *S. suis* cells.

Peripheral blood mononuclear cells were also obtained to determine the frequencies of unspecific IFN-γ-secreting-cells (IFNγ-SC) by ELISPOT after PHA *in vitro* stimulation (1).

Results and Discussion

As it was expected, neutrophils demonstrated higher phagocytic capacity than macrophages in all the analyzed samples. The proportion of phagocytic neutrophils and the average PhI for those cells were always 2 to 2.5 higher than the values of these parameters for macrophages. No differences in the phagocytic capacity of neutrophils were noticed related to the age of the pigs. This finding is in agreement with other studies (2) that showed that the maturation of pigs affected intracellular killing but no the phagocytic ability of neutrophils.

Regarding macrophages, the percentage of phagocytic cells decreased at 8 weeks of age (Figure 1). At that time, the ratio PhI: percentage of macrophages that effectively phagocited *S. suis* cells showed that macrophages were more efficiency to phagocyte the bacteria (Figure 2). These results suggest that as pig increases in age, a proportion of macrophages mature by increasing its ability to ingest bacteria although the total number of phagocytic macrophages decreased.

Figure 1. Phagocytic capacity of macrophages

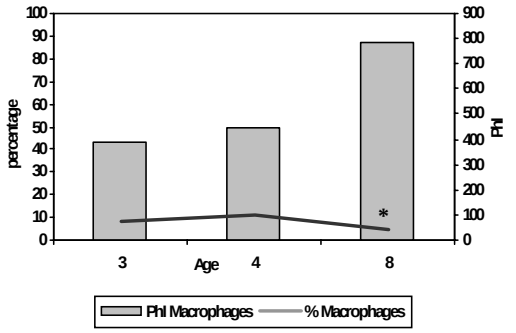
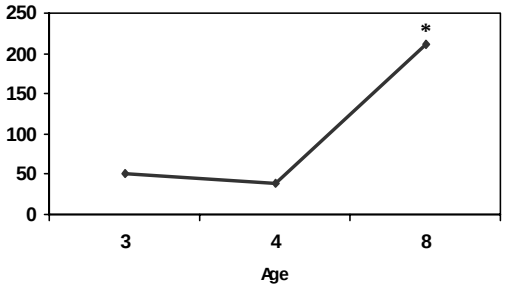


Figure 2. Ratio PhI: percentage of macrophages.



(*) $p<0.05$

As we had reported before (1), the unspecific response of T cells analyzed after stimulation with a mitogen showed that frequencies of IFNγ-SC significantly increased with age ($p<0.05$). Taken together, these results indicate that maturation of pigs affects differently to macrophages and neutrophils. This can have important implications for both the innate and the adaptative immune responses at different ages.

References

1. Díaz I. & Mateu E. (2005). Vet. Imm. Immunopath. 106, 107-112.
2. Mainor J. M. et al. (2002). Vet. Imm. Immunopath. 89, 175-186.

Bibliografía

El verdadero modo de no saber nada es aprenderlo todo a la vez

George Sand

8. BIBLIOGRAFÍA

Abbas AK, Lichtman AH, Pober JS (2000) Cytokines. In: Cellular and Molecular Immunology, 4th ed. Philadelphia. p235–69.

Albina E, Vannier P, Madec F, Cariolet R, Torrison J (1994) Persistence of the porcine epidemic abortion and respiratory syndrome (PEARS) virus in infected pigs and farm units. In: Proceedings of the 13th International Pig Veterinary Society Congress, p53.

Albina E, Carrat C, Charley B (1998a) Interferon-alpha response to swine arterivirus (PoAV), the porcine reproductive and respiratory syndrome virus. J Interferon Cytokine Res 18: 485-90.

Albina E, Piriou L, Hutet E, Cariolet R, L'Hospitalier R (1998b) Immune responses in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). Vet Immunol Immunopathol 61:49-66.

Allende R, Lewis TL, Lu Z, Rock DL, Kutish GF, Ali A, Doster AR, Osorio FA (1999) North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J Gen Virol 80:307-15.

Allende R, Kutish GF, Laegreid W, Lu Z, Lewis TL, Rock DL, Friesen J, Galeota JA, Doster AR, Osorio FA (2000) Mutations in the genome of porcine reproductive and respiratory syndrome virus responsible for the attenuation phenotype. Arch Virol 145:1149-61.

Andreyev VG, Wesley RD, Mengeling WL, Vorwald AC, Lager KM (1997) Genetic variation and phylogenetic relationships of 22 porcine reproductive and respiratory syndrome virus (PRRSV) field strains based on sequence analysis of open reading frame 5. Arch Virol 142:993-1001.

Ansari IH, Kwon B, Osorio FA, Pattnaik AK (2006) Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. J Virol 80:3994-4004.

Bach JF (2003) Regulatory T cells under scrutiny. Nat Rev Immunol 3:189-98.

Baker B, Thacker E, Thacker B, Vincent A (1999) A preliminary investigation into possible PRRSV energy induction from repeated immunization with a modified live vaccine. In: Proceedings of the 26th Allen D. Leman Swine Conference, p31.

Balasuriya UB & MacLachlan NJ (2004) The immune response to equine arteritis virus: potential lessons for other arteriviruses. Vet Immunol Immunopathol 102:107-29.

Barfoed AM, Blixenkrone-Moller M, Jensen MH, Botner A, Kamstrup S (2004) DNA vaccination of pigs with open reading frame 1-7 of PRRS virus. Vaccine 22:3628-41.

Baron T, Albina E, Leforban Y, Madec F, Guilmoto H, Plana Duran J, Vannier P (1992) Report on the first outbreaks of the porcine reproductive and respiratory syndrome (PRRS) in France. Diagnosis and viral isolation. Ann Rech Vet 23:161-6.

Bassaganya-Riera J, Thacker BJ, Yu S, Strait E, Wannemuehler MJ, Thacker EL (2004) Impact of immunizations with porcine reproductive and respiratory syndrome virus on lymphoproliferative recall responses of CD8+ T cells. Viral Immunol. 17:25-37.

Bastos RG, Dellagostin OA, Barletta RG, Doster AR, Nelson E, Zuckermann F, Osorio FA (2004) Immune response of pigs inoculated with Mycobacterium bovis BCG expressing a truncated form of GP5 and M protein of porcine reproductive and respiratory syndrome virus. Vaccine 22:467-74.

Bautista EM, Meulenberg JJM, Choi CS, Molitor TW (1996) Structural polypeptides of the American (VR-2332) strain of porcine reproductive and respiratory syndrome virus. Arch Virol 141:1357-65.

Bautista EM & Molitor TW (1997) Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. Viral Immunol 10:83-94.

Bautista EM & Molitor TW (1999) IFN gamma inhibits porcine reproductive and respiratory syndrome virus replication in macrophages. Arch Virol 144:1191-200.

Bautista EM, Suarez P, Molitor TW (1999) T cell responses to the structural polypeptides of porcine reproductive and respiratory syndrome virus. Arch Virol 144:117-34.

Becker JC, Czerny C, Brocker EB (1994) Maintenance of clonal anergy by endogenously produced IL-10. Int Immunol 6:1605-12.

Benfield DA, Nelson E, Collins JE, Harris L, Goyal SM, Robison D, Christianson WT, Morrison RB, Gorcyca D, Chladek D (1992) Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J Vet Diagn Invest 4:127- 33.

Benfield DA, Christopher-Hennings J, Nelson EA, Rowland RR, Nelson JK, Chase C CL, Rossow KD, Collins JE (1997) Persistent fetal infection of porcine reproductive and respiratory syndrome (PRRS) virus. In: Proceedings of the 28th Annual Meeting American Association Swine Practitioners, p455-8.

Benfield DA, Collins JE, Dee SA, Halbur PG, Joo HS, Lager KM, Mengeling WL, Murtaugh MP, Rossow KD, Stevenson GW, Zimmerman JJ (1999) Porcine reproductive and respiratory syndrome. In: Diseases of Swine, 8th ed. Ed: Straw BE, D'Allaire S, Mengeling WL, Taylor DJ. Iowa, p201-232.

Benfield D, Nelson J, Rossow K (2000) Diagnosis of persistent or prolonged porcine reproductive and respiratory syndrome virus infections. Vet Rec 31:71.

Benson JE, Yaeger MJ, Ford SP (2001) Effect of porcine reproductive and respiratory syndrome virus infection on the ovary and progesterone levels in third trimester pregnant sows. Theriogenology 56:777-85.

Bloemraad M, de Kluijver EP, Petersen A, Burkhardt GE, Wensvoort G (1994) Porcine reproductive and respiratory syndrome: Temperature and pH stability of Lelystad virus and its survival in tissue specimens from viraemic pigs. Vet Microbiol 42:361-71.

Botner A, Nielsen J, Bille-Hansen V (1994) Isolation of porcine reproductive and respiratory syndrome (PRRS) virus in a Danish swine herd and experimental infection of pregnant gilts with the virus. Vet Microbiol 40:351-60.

Botner A, Strandbygaard B, Sorensen KJ, Have P, Madsen K, Madsen E, Alexandersen S (1997) Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. Vet Rec 8:497-9.

Brady MT, McDonald AJ, Rowan AG, Mills KH (2003) Hepatitis C virus non-structural protein suppresses Th1 responses by stimulating IL-10 production from monocytes. Eur J Immunol 33:3448-57.

Buddaert, W, Van Reeth K, Pensaert M (1998) In vivo and in vitro interferon studies with the porcine reproductive and respiratory syndrome virus. Adv Exp Med Biol 440:461-7.

Cancel-Tirado SM, Evans RB, Yoon KJ (2004) Monoclonal antibody analysis of porcine reproductive and respiratory syndrome virus epitopes associated with antibody-dependent enhancement and neutralization of virus infection. Vet Immunol Immunopathol 2004 8:249-62.

Carman S, Sanford SE, Dea S (1995) Assessment of seropositivity to porcine reproductive and respiratory syndrome (PRRS) virus in swine herds in Ontario-1978 to 1982. Can Vet J 36:776-7.

Carter QL, Curiel RE (2005) Interleukin-12 (IL-12) ameliorates the effects of porcine respiratory and reproductive syndrome virus (PRRSV) infection. Vet Immunol Immunopathol 15:105-18.

Cavanagh, D (1997) Nidovirales: A new order comprising Coronaviridae and Arteriviridae. Arch Virol 142:629-33.

Chan SH, Perussia B, Gupta JW, Kobayashi M, Pospisil M, Young HA, Wolf SF, Young D, Clark SC, Trinchieri G (1991) Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J Exp Med 173:869–79.

Chang CC, Yoon KJ, Zimmerman JJ, Harmon KM, Dixon PM, Dvorak CM, Murtaugh MP (2002) Evolution of porcine reproductive and respiratory syndrome virus during sequential passages in pigs. J Virol 76:4750-63.

Charerntantanakul W, Platt R, Johnson W, Roof M, Vaughn E, Roth JA (2006) Immune responses and protection by vaccine and various vaccine adjuvant candidates to virulent porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 109:99-115.

Chen Z, Li K, Rowland RR, Plagemann PG (1998) Neuropathogenicity and susceptibility to immune response are interdependent properties of lactate dehydrogenase-elevating virus (LDV) and correlate with the number of N-linked polylactosaminoglycan chains on the ectodomain of the primary envelope glycoprotein. Adv Exp Med Biol 440:583-92.

Chiou MT, Jeng CR, Chueh LL, Cheng CH, Pang VF (2000) Effects of porcine reproductive and respiratory syndrome virus (isolate tw91) on porcine alveolar macrophages in vitro. Vet Microbiol 71:9-25.

Choi C, Christianson W, Collins J, Joo HS, Molitor T (1992) Antibody dependent enhancement of SIRS virus replication. In: American Association of Swine Practitioners Newsletter 4:30.

Choi C, Gustafson K, Chinsakchai S, Hill H, Molitor T (1994) Heterogeneity of porcine alveolar macrophage subpopulations: Immune functions and susceptibility to PEARS virus. In: Proceedings of the International Pig Veterinary Society Congress, p97.

Choi C & Chae C (2002) Expression of tumour necrosis factor-alpha is associated with apoptosis in lungs of pigs experimentally infected with porcine reproductive and respiratory syndrome virus. Res Vet Sci 72:45-9.

Christianson WT, Collins JE, Pijoan C, Joo HS, Benfield DA, McCullough SJ (1991) Swine infertility and respiratory syndrome. Pig Vet J 27:9-12.

Christianson WT, Collins JE, Benfield DA, Harris L, Gorcyca DE, Chladek DW, Morrison RB, Joo HS (1992) Experimental reproduction of swine infertility and respiratory syndrome in pregnant sows. Am J Vet Res 53:485-8.

Christianson WT, Choi CS, Collins JE, Molitor TW, Morrison RB, Joo HS (1993) Pathogenesis of porcine reproductive and respiratory syndrome virus infection in mid-gestation sows and fetuses. Can J Vet Res 57:262-8.

Christopher-Hennings J, Nelson EA, Nelson JA, Hines RJ, Swenson SL, Hill HT, Zimmerman JJ, Katz JB, Yaeger MJ, Chase CCL, Benfield DA (1995) Detection of porcine reproductive and respiratory syndrome virus in boar semen by PCR. J Clin Microbiol 33:1730-4.

Christopher-Hennings J, Nelson EA, Nelson JK, Rossow KD, Shivers JL, Yaeger MJ, Chase CC, Garduno RA, Collins JE, Benfield DA (1998) Identification of porcine reproductive and respiratory syndrome virus in semen and tissues from vasectomized and nonvasectomized boars. Vet Pathol 35:260-7.

Christopher-Hennings J, Holler LD, Benfield DA, Nelson EA (2001) Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral blood mononuclear cells, and tissues from Yorkshire, Hampshire, and Landrace boars. J Vet Diagn Invest 13:133-42.

Christopher-Hennings J, Faaberg KS, Murtaugh MP, Nelson EA, Roof MB, Vaughn EM, Yoon KJ, Zimmerman J (2002) Porcine reproductive and respiratory syndrome (PRRS) diagnostics: Interpretation and limitations. J Swine Health Prod 10:213-8.

Coffer PJ & Burgering BM (2004) Forkhead-box transcription factors and their role in the immune system. Nat Rev Immunol 4:889-99.

Collins JE (1991) Diagnostic note: newly recognized respiratory syndromes in North American swine herds. American Association of Swine Practitioners Newsletter 3:7-11.

Collins JE, Benfield DA, Christianson WT, Harris L, Hennings JC, Shaw DP, Goyal SM, McCullough S, Morrison RB, Joo HS (1992) Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J Vet Diagn Invest 4:117-26.

Conzelmann KK, Visser N, Van Woensel P, Thiel HJ (1993) Molecular characterization of porcine reproductive and respiratory sydnrome virus, a member of the arterivirus group. Virology 193:329-39.

Cromwijk W (1991) The 'new' pig disease – further observations in Dutch herds. In: PRRS (the new pig disease). A report on the seminar held in Brussels on 4-5 November 1991 and organized by the European Commission (Directorate General for Agriculture), p20-7.

Czerkinsky C, Andersson G, Ekre HP, Nilsson LA, Klareskog L, Ouchterlony O (1988) Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. J Immunol Methods 110:29–36.

Damoiseaux J (2006) Regulatory T cells: back to the future. Neth J Med 64:4-9.

Dea S, Bilodeau R, Athanaseous R, Sauvageau RA, Martineau GP (1992) PRRS syndrome in Quebec: isolation of a virus serologically related to Lelystad virus. Vet Rec 130:167.

Dea S, Sawyer N, Alain R, Athanassious R (1995) Ultrastructural characteristics and morphogenesis of porcine reproductive and respiratory syndrome virus propagated in the highly permissive MARC-145 cell clone. Adv Exp Med Biol 380:95-8.

Dea S, Gagnon CA, Mardassi H, Milane G (1996) Antigenic variability among North American and European strains of porcine reproductive and respiratory syndrome virus as defined by monoclonal antibodies to the matrix protein. J Clin Microbiol 34:1488-93.

Dee SA & Joo HS (1994) Prevention of the spread of porcine reproductive and respiratory syndrome virus in endemically infected pig populations using nursery depopulation. Vet Rec 135:6-9.

Dee SA, Deen J, Rossow K, Wiese C, Otake S, Joo HS, Pijoan C (2002) Mechanical transmission of porcine reproductive and respiratory syndrome virus throughout a coordinated sequence of events during cold weather. Can J Vet Res 66:232-9.

Dee SA, Deen J, Rossow K, Weise C, Eliason R, Otake S, Joo S, Pijoan C (2003) Mechanical transmission of porcine reproductive and respiratory syndrome virus throughout a coordinated sequence of events during warm weather. Can J Vet Res 67:12-9.

Dee SA, Deen J, Otake S, Pijoan C (2004) An experimental model to evaluate the role of transport vehicles as a source of transmission of porcine reproductive and respiratory syndrome virus to susceptible pigs. Can J Vet Res 68:128-33.

Dee SA, Deen J, Burns D, Douthit G, Pijoan C (2005) An evaluation of disinfectants for the sanitation of porcine reproductive and respiratory syndrome virus-contaminated transport vehicles at cold temperatures. Can J Vet Res 69:64-70.

De Jong MF, van't Veld P, Cromwijk W (1991) The epidemiology of PRRS in The Netherlands. In: PRRS (the new pig disease). A report on the seminar held in Brussels on 4-5 November 1991 and organized by the European Commission (Directorate General for Agriculture), p2-7.

Delputte PL, Vanderheijden N, Nauwynck HJ, Pensaert MB (2002) Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. J Virol 76:4312-20.

Delputte PL, Meerts P, Costers S, Nauwynck HJ (2004) Effect of virus-specific antibodies on attachment, internalization and infection of porcine reproductive and respiratory syndrome virus in primary macrophages. Vet Immunol Immunopathol 102:179-88.

Desrosiers R & Boutin M (2002) An attempt to eradicate porcine reproductive and respiratory syndrome virus (PRRSV) after an outbreak in a breeding herd: eradication strategy and persistence of antibody titers in sows. Swine Health Prod 10:23-5.

Dewey C, Charbonneau G, Carman S, Hamel A, Nayar G, Friendship R, Eernisse K, Swenson S (2000) Lelystad-like strain of porcine reproductive and respiratory syndrome virus (PRRSV) identified in Canadian swine. Can Vet J 41:493-4.

Dewey CE, Wilson S, Buck P, Leyenaar JK (2004) Effects of porcine reproductive and respiratory syndrome vaccination in breeding-age animals. Prev Vet Med 62:299-307.

Diaz I & Mateu E (2005) Use of ELISPOT and ELISA to evaluate IFN-gamma, IL-10 and IL-4 responses in conventional pigs. Vet Immunol Immunopathol 106:107-12.

Dittmer U, He H, Messer RJ, Schimmer S, Olbrich AR, Ohlen C, Greenberg PD, Stromnes IM, Iwashiro M, Sakaguchi S, Evans LH, Peterson KE, Yang G, Hasenkrug KJ (2004) Functional impairment of CD8(+) T cells by regulatory T cells during persistent retroviral infection. Immunity 20:293-303.

Doetze A, Satoguina J, Burchard G, Rau T, Loliger C, Fleischer B, Hoerauf A (2000) Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift. Int Immunol 12:623-30.

Domingo E & Holland JJ (1997) RNA virus mutations and fitness for survival. Annu Rev Microbiol 51:151-78.

Done SH & Paton DJ (1995) Porcine reproductive and respiratory syndrome: Clinical disease, pathology and immunosupression. Vet Rec 136:32-5.

Done SH, Paton DJ, White MEC (1996) Porcine reproductive and respiratory syndrome (PRRS): A review, with emphasis on pathological, virological, and diagnostic aspects. Br Vet J 152:153-74.

Duan X, Nauwynck HJ, Pensaert MB (1997) Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). Arch Virol 142:2483-97.

Fairbanks K, Chase C, Benfield DA (2002) Tonsil biopsies and polymerase chain reaction assay for detection of breeding age gilts persistently infected with porcine reproductive and respiratory syndrome virus. J Swine Health Prod 10:87-8.

Feitsma H, Grooten HJ, Schie FWV, Colenbrander B (1992) The effect of porcine epidemic abortion and respiratory syndrome (PEARS) on sperm production. In: Proceedings of the 12th International Congress on Animal Reproduction, p1710-2.

Ferrin NH, Fang Y, Johnson CR, Murtaugh MP, Polson DD, Torremorell M, Gramer ML, Nelson EA (2004) Validation of a blocking enzyme-linked immunosorbent assay for detection of antibodies against porcine reproductive and respiratory syndrome virus. Clin Diagn Lab Immunol 11:503-14.

Forsberg R, Oleksiewicz MB, Petersen AM, Hein J, Botner A, Storgaard T (2001) A molecular clock dates the common ancestor of European-type porcine reproductive and respiratory syndrome virus at more than 10 years before the emergence of disease. Virology 289:174-9.

Forsberg R, Storgaard T, Nielsen HS, Oleksiewicz MB, Cordioli P, Sala G, Hein J, Botner A (2002) The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. Virology 299:38-47.

Foss DL & Murtaugh MP (1999) Mucosal immunogenicity and adjuvanticity of cholera toxin in swine. Vaccine 17788-801.

Foss DL, Zilliox MJ, Meier W, Zuckermann F, Murtaugh MP (2002) Adjuvant danger signals increase the immune response to porcine reproductive and respiratory syndrome virus. Viral Immunol 15:557-66.

Geue A (1995) Untersuchungen zur Pravalenz und Inzidenz des Porcine Reproductive and Respiratory Syndrome (PRRS) in einem Kreis Schleswig-Holstein. Fachbereich Veterinarmedizin, Freie Universitat, Berlin, Germany.

Gilbert SA, Larochelle R, Magar R, Cho HJ, Deregt D (1997) Typing of porcine reproductive and respiratory syndrome viruses by a multiplex PCR assay. J Clin Microbiol 35:264-7.

Goldberg TL, Hahn EC, Weigel RM, Scherba G (2000) Genetic, geographical and temporal variation of porcine reproductive and respiratory syndrome virus in Illinois. J Gen Virol 81:171-9.

Goldberg TL, Lowe JF, Milburn SM, Firkins LD (2003) Quasispecies variation of porcine reproductive and respiratory syndrome virus during natural infection.Virology 317:197-207.

Gorcyca D, Schlesinger K, Chladek D, Behan W, Polson D, Roof M, Doitchenoff D (1995) RespPRRS: A new tool for the prevention and control of PRRS in pigs. In: Proceedings of 26th Annual Meeting American Associaton Swine Pract, p1-22.

Goyal SM (1993) Porcine reproductive and respiratory syndrome: Review article. J Vet Diagn Invest 5:656-64.

Gradil C, Dubuc C, Eaglesome MD (1996) Porcine reproductive and respiratory syndrome virus: Seminal transmission. Vet Rec 138:521-2.

Grebennikova TV, Clouser DF, Vorwald AC, Musienko MI, Mengeling WL, Lager KM, Wesley RD, Biketov SF, Zaberezhny AD, Aliper TI, Nepoklonov EA (2004) Genomic characterization of virulent, attenuated, and revertant passages of a North American porcine reproductive and respiratory syndrome virus strain. Virology 321:383-90.

Groux H, Bigler M, de Vries JE, Roncarolo MG (1996) Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. J Exp Med 184:19-29.

Groux H, Bigler M, de Vries JE, Roncarolo MG (1998) Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. J Immunol 160:3188-93.

Halbur PG, Paul PS, Meng XJ, Lum MA, Andrews JJ, Rathje JA (1996) Comparative pathogenicity of nine U.S. porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a 5-week-old cesarean-derived-colostrum deprived pig model. J Vet Diagn Invest 8:11-20.

Halbur PG, Rothschild MF, Thacker BJ, Meng XJ, Paul PS, Bruna JD (1998) Differences in susceptibility of Duroc, Hampshire, and Meishan pigs to infection with a high virulence strain (VR2385) of porcine reproductive and respiratory syndrome virus (PRRSV). J Anim Breed Genet 115:181-9.

Halbur PG (2003) Factors that influence the severity of clinical disease. In: PRRS Compendium, 2d edition. Ed: Zimmerman JJ, Yoon KJ. Iowa, p17-25.

Hanada K, Suzuki Y, Nakane T, Hirose O, Gojobori T (2005) The origin and evolution of porcine reproductive and respiratory syndrome viruses. Mol Biol E 22:1024–31.

Haynes JS, Halbur PG, Sirinarumitr T, Paul PS, Meng XJ, Huffman EL (1997) Temporal and morphologic characterization of the distribution of porcine reproductive and respiratory syndrome virus (PRRSV) by in situ hybridization in pigs infected with isolates of PRRSV that differ in virulence. Vet Pathol 34:39-43.

Henryon M, Berg P, Jensen J, Andersen S (2001) Genetic variation for resistance to clinical and subclinical diseases exists in growing pigs. Animal Science: an International Journal of Fundamental and Applied Research 73: 375-87

Hermann JR, Munoz-Zanzi CA, Roof MB, Burkhart K, Zimmerman JJ (2005) Probability of porcine reproductive and respiratory syndrome (PRRS) virus infection as a function of exposure route and dose. Vet Microbiol 110:7-16.

Hill H (1990) Overview and history of Mystery Swine Disease (Swine infertility/respiratory syndrome). In: Proceedings of Mystery Swine Disease Committee Meeting, Livestock Conservation Institute, Denver, p29-31

Hirose O, Kudo H, Yoshizawa S, Hiroike T, Nakane T (1995) Prevalence of porcine reproductive and respiratory syndrome virus in Chiba prefecture. J Jpn Vet Med Assoc 48:650-3.

Hopper SA, White ME, Twiddy N (1992) An outbreak of blue-eared pig disease (porcine reproductive and respiratory syndrome) in four pig herds in Great Britain. Vet Rec 131:140-4.

Hori S, Sakaguchi S (2004) Foxp3: a critical regulator of the development and function of regulatory T cells. Microbes Infect 6:745-51.

Horter DC, Pogranichniy RM, Chang CC, Evans RB, Yoon KJ, Zimmerman JJ (2002) Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection. Vet Microbiol 86:3213-8.

Hu BT & Insel RA (1999) Up-regulation of telomerase in human B lymphocytes occurs independently of cellular proliferation and with expression of the telomerase catalytic subunit. Eur J Immunol 29:3745-53.

Hurd HS, Bush EJ, Losinger W, Corso B, Zimmerman J, Wills B, Swenson S, Pyburn D, Yeske P, Burkgren T (2001) Outbreaks of porcine reproductive failure: Report on a collaborative field investigation. J Swine Health Prod 9:103-8.

Itoh K & Hirohata S (1995) The role of IL-10 in human B cell activation, proliferation, and differentiation. J Immunol 154:4341-50.

Iwashiro M, Messer RJ, Peterson KE, Stromnes IM, Sugie T, Hasenkrug KJ (2001) Immunosuppression by CD4+ regulatory T cells induced by chronic retroviral infection. Proc Natl Acad Sci USA 98:9226-30

Joo HS, Park BK, Dee SA, Pijoan C (1997) Indirect fluorescent IgM antibody response of pigs infected with porcine reproductive and respiratory syndrome syndrome virus. Vet Microbiol 55:303-7.

Joo HS, Direksin K, Johnson C, Lee W, DeBuse N (1999) PRRSV virus serum neutralization antibody as a measure of protection. In: Proceedings of the 26th Allen D. Leman Swine Conference, p183–6.

Jusa ER, Inaba Y, Kohno M, Mashimo H, Hirose O (1996) Hemagglutination with porcine reproductive and respiratory syndrome virus. J Vet Med Sci 58:521-7.

Kapur V, Elam MR, Pawlovich TM, Murtaugh MP (1996) Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J Gen Virol 77:1271-6.

Keay S, Moreau IA, Provis P (2002) A retrospective study of PRRS diagnostic results from routine serologic testing of seven PRRS naïve herds. Proc Annu Meet Am Assoc Swine Vet p147-8.

Keffaber KK (1989) Reproductive failure of unknown etiology. American Association of Swine Practitioners Newsletter 1:1-10.

Kim HS, Kwang, J, Yoon IJ, Joo, HS, Frey ML (1993) Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogenous subpopulation of MA-104 cell line. Arch Virol 133:477-83.

Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med **170**:827–46.

Kranker S, Nielsen J, Bille-Hansen V, Bøtner A (1998) Experimental inoculation of swine at various stages of gestation with a Danish isolate of porcine reproductive and respiratory syndrome virus (PRRSV). Vet Microbiol 61:21-31.

Kristensen CS, Bøtner A, Angen Ø, Sørensen V, Jorsal SE, Takai H, Barfod K, Nielsen JP (2002) Airborne transmission of *A. pleuropneumoniae* and PRRS virus between pig units. In: Proceedings of the 17th International Pig Veterinary Society Congress, p272.

Kwang J, Zuckermann F, Ross G, Yang S, Osorio F, Liu W, Low S (1999) Antibody and cellular immune responses of swine following immunization with plasmid DNA encoding the PRRS virus ORFs 4, 5, 6 and 7. Res Vet Sci 67:199-201.

Labarque G, Nauwynck HJ, Van Reeth K, Pensaert MB (2000) Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. J Gen Virol 81:1327-34.

Labarque G, Van Gucht S, Van Reeth K, Nauwynck H, Pensaert M (2003) Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines. Vet Microbiol 95:187-97.

Labarque G, Reeth KV, Nauwynck H, Drexler C, Van Gucht S, Pensaert M (2004) Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. Vaccine 22:4183-90.

Lager KM, Mengeling WL (1995) Pathogenesis of in utero infection in porcine fetuses with porcine reproductive and respiratory syndrome virus. Can J Vet Res. 59:187-92.

Lager KM, Mengeling WL, Brockmeier SL (1996) Effect of post-coital intrauterine inoculation of porcine reproductive and respiratory syndrome virus on conception in gilts. Vet Rec 138:227-8.

Lager KM, Mengeling WL, Brockmeier SL (1997a) Homologous challenge of porcine reproductive and respiratory syndrome virus immunity in pregnant swine. Vet Microbiol 58:113-25.

Lager KM, Mengeling WL, Brockmeier SL (1997b) Duration of homologous porcine reproductive and respiratory syndrome virus immunity in pregnant swine. Vet Microbiol. 58:127-33.

Lager KM, Halbur PG, Mengeling WL (1998) Evaluation of the pathogenic effects of an 'atypical PRRS' virus isolate in pregnant gilts. In: Proceedings of the 15th International Pig Veterinary Society Congress, p137.

Lager KM, Mengeling WL, Brockmeier SL (1999) Evaluation of protective immunity in gilts inoculated with the NADC-8 isolate of porcine reproductive and respiratory syndrome virus (PRRSV) and challenge-exposed with an antigenically distinct PRRSV isolate. Am J Vet Res 60:1022-7.

Lamontagne L, Page C, Larochelle R, Longtin D, Magar R (2001) Polyclonal activation of B cells occurs in lymphoid organs from porcine reproductive and respiratory syndrome virus (PRRSV)-infected pigs. Vet Immunol Immunopathol 82:165-82.

Lamontagne L, Page C, Larochelle R, Magar R (2003) Porcine reproductive and respiratory syndrome virus persistence in blood, spleen, lymph nodes, and tonsils of experimentally infected pigs depends on the level of CD8high T cells. Viral Immunol 16:395-406.

Lee SM, Schommer SK, Kleiboeker SB (2004) Porcine reproductive and respiratory syndrome virus field isolates differ in in vitro interferon phenotypes. Vet Immunol Immunopathol 102:217-31.

Le Gall A, Legeay O, Bourhy H, Arnauld C, Albina E, Jestin A (1998) Molecular variation in the nucleoprotein gene (ORF7) of the porcine reproductive and respiratory syndrome virus (PRRSV). Virus Res 54:9-21.

Le Potier M-F, Blanquefort P, Morvan E, Albina E (1997) Results of a control programme for the porcine reproductive and respiratory syndrome in the French "Pays de la Loire" region. Vet Microbiol 55:355-60.

Li K, Chen Z, Plagemann P (1998) The neutralization epitope of lactate dehydrogenase-elevating virus is located on the short ectodomain of the primary envelope glycoprotein. Virology 242:239-45.

Lindhaus W, Lindhaus B (1991) Rätselhafte Schweinekrankheit. Praktische Tierarztliche 5:423-5.

Loemba HD, Mounir S, Mardassi H, Archambault D, Dea S (1996) Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. Arch Virol 141:751-61.

Lohse L, Nielsen J, Eriksen L (2004) Temporary CD8+ T-cell depletion in pigs does not exacerbate infection with porcine reproductive and respiratory syndrome virus (PRRSV). Viral Immunol 17: 594-603.

López OJ & Osorio FA (2004) Role of neutralizing antibodies in PRRSV protective immunity. Vet Immunol Immunopathol 102:155-63.

López-Fuertes L, Domenech N, Alvarez B, Ezquerra A, Dominguez J, Castro JM, Alonso F (1999) Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. Virus Res 64:33-42.

López-Fuertes L, Campos E, Domenech N, Ezquerra A, Castro JM, Dominguez J, Alonso F (2000) Porcine reproductive and respiratory syndrome (PRRS) virus down-modulates TNF-alpha production in infected macrophages. Virus Res 69:41-6.

Loula T (1991) Mystery pig disease. Agri Prac 12:23-34.

Lowe JE, Husmann R, Firkins LD, Zuckermann FA, Goldberg TL (2005) Correlation of cell-mediated immunity against porcine reproductive and respiratory syndrome virus with protection against reproductive failure in sows during outbreaks of porcine reproductive and respiratory syndrome in commercial herds. J Am Vet Med Assoc 226:1707-11.

Lu C, Lim KI, Hahn TW (1999) Seroprevalence of ELISA antibody to porcine reproductive and respiratory syndrome virus in Korea. Annals of Animal Resources Science 10:127-37.

Madsen KG, Hansen CM, Madsen ES, Strandbygaard B, Botner A, Sorensen KJ (1998) Sequence analysis of porcine reproductive and respiratory syndrome virus of the American type collected from Danish swine herds. Arch Virol 143:1683-700.

Maes D (1997) Descriptive epidemiological aspects of the seroprevalence of five respiratory disease agents in slaughter pigs from fattening herds. Epidémiol Santé Anim 31-32:05.B.19.

Magar R, Robinson Y, Dubuc C, Larochelle R (1995) Isolation and experimental oral transmission in pigs of a porcine reproductive and respiratory syndrome virus isolate. Adv Exp Med Biol 380:139-44.

Magar R, Larochelle R, Nelson EA, Charreyre C (1997) Differential reactivity of a monoclonal antibody directed to the membrane protein of porcine reproductive and respiratory syndrome virus. Can J Vet Res 61:69-71.

Magar R, Larochelle R (2004) Evaluation of the presence of porcine reproductive and respiratory syndrome virus in pig meat and experimental transmission following oral exposure. Can J Vet Res 68:259-66.

Mardassi H, Athanassious R, Mounir S, Dea S (1994) Porcine reproductive and respiratory syndrome virus: morphological, biochemical and serological characteristics of Quebec isolates associated with acute and chronic outbreaks of porcine reproductive and respiratory syndrome. Can J Vet Res 58:55-64.

Mardassi H, Massie B, Dea S (1996) Intracellular synthesis, processing, and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. Virology 221:98-112.

Mateu E, Martin M, Vidal D (2003) Genetic diversity and phylogenetic analysis of glycoprotein 5 of European-type porcine reproductive and respiratory virus strains in Spain. J Gen Virol 84:529-34.

Mateu E, Diaz I, Darwich L, Casal J, Martin M, Pujols J (2006) Evolution of ORF5 of Spanish porcine reproductive and respiratory syndrome virus strains from 1991 to 2005. Virus Res 115:198-206.

Mateu E, Tello M, Coll A, Casal J, Martin M (en prensa) Comparision of three ELISAs for the diagnosis of porcine reproductive and respiratory syndrome. Vet Rec.

McGuirk P & Mills KH (2002) Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. Trends Immunol 23:450-5.

Medveczky I, Kulcsár G, Makranszki L, Glávits R, Gorcyca D, Schütz B (2002) Efficacy of a PRRS modified live virus vaccine (US-strain) against the heterologous infection by a virulent PRRS virus (EU-strain): reproductive performance. In: Proceedings of the 17th International Pig Veterinary Society Congress, p377.

Meier W, Wheeler J, Husmann RJ, Osorio F, Zuckermann FA (2000) Characteristics of the immune response of pigs to PRRS virus. Vet Res 31:41.

Meier WA, Galeota J, Osorio FA, Husmann RJ, Schnitzlein WM, Zuckermann FA (2003) Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology 309:18-31.

Meier WA, Husmann RJ, Schnitzlein WM, Osorio FA, Lunney JK, Zuckermann FA (2004) Cytokines and synthetic double-stranded RNA augment the T helper 1 immune response of swine to porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 102:299-314.

Melnichouk O, Dewey CE, Friendship RM, Haydon D (en prensa) Seroepidemiological study of porcine reproductive and respiratory syndrome (PRRS) virus infection patterns by age influence in pigs on seven commercial farms in Ontario, Canada. Prev Vet Med.

Meng XJ, Paul PS, Halbur PG (1994) Molecular cloning and nucleotide sequencing of the 3'-terminal genomic RNA of the porcine reproductive and respiratory syndrome virus. J Gen Virol 75:1795-801.

Meng XJ, Paul PS, Halbur PG, Lum MA (1996) Characterization of a high-virulence US isolate of porcine reproductive and respiratory syndrome virus in a continuous cell line, ATCC CRL11171. J Vet Diagn Invest 8:374-81.

Meng XJ (2000) Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet Microbiol 74:309-29.

Mengeling WL, Lager KM, Vorwald AC (1994) Temporal characterization of transplacental infection of porcine fetuses with porcine reproductive and respiratory syndrome virus. Am J Vet Res 55:1391-8.

Mengeling WL, Lager KM, Vorwald AC (1995) Diagnosis of porcine reproductive and respiratory syndrome. J Vet Diagn Invest 7:3-16.

Mengeling WL, Vorwald AC, Lager KM, Brockmeier SL (1996) Comparison among strains of porcine reproductive and respiratory syndrome virus for their ability to cause reproductive failure. Am J Vet Res 57:834-9.

Mengeling WL, Lager KM, Vorwald AC (1999) Safety and efficacy of vaccination of pregnant gilts against porcine reproductive and respiratory syndrome. Am J Vet Res. 60:796-801.

Mengeling WL, Lager KM, Vorwald AC, Clouser DF (2003a) Comparative safety and efficacy of attenuated single-strain and multi-strain vaccines for porcine reproductive and respiratory syndrome. Vet Microbiol 93:25-38.

Mengeling WL, Lager KM, Vorwald AC, Koehler KJ (2003b) Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. Vet Microbiol 93:13-24.

Meredith MJ (1992) Review of porcine reproductive and respiratory syndrome. Cambridge, UK: Pig Disease Information Centre, p1-24.

Meulenberg JJ, Hulst MM, de Meijer EJ, Moonen PL, den Besten A, de Kluyver EP, Wensvoort G, Moormann RJ (1993) Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192:62-72.

Meulenberg JJ, Hulst MM, de Meijer EJ, Moonen PL, den Besten A, de Kluyver EP, Wensvoort G, Moormann RJ (1994) Lelystad virus belongs to a new virus family, comprising lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus. Arch Virol Suppl 9:441-8.

Meulenberg JJ, Petersen-den Besten A, de Kluyver EP, Moormann RJ, Schaaper WM, Wensvoort G (1995a) Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. Virology 206:155-63.

Meulenberg JJ, Petersen-den Besten A, de Kluyver EP, Moormann RJ, Schaaper WM, Wensvoort G (1995b) Characterization of structural proteins of Lelystad virus. Adv Exp Med Biol 380:271-6.

Meulenberg JJ, Bende RJ, Pol JM, Wensvoort G, Moormann RJ (1995c) Nucleocapsid protein N of Lelystad virus: expression by recombinant baculovirus, immunological properties, and suitability for detection of serum antibodies. Clin Diagn Lab Immunol. 2:652-6.

Meulenberg JJ, den Besten A (1996) Identification and characterization of a sixth structural protein of Lelystad virus: the glycoprotein GP2 encoded by ORF 2 is incorporated in virus particles. Virology 225:44-51.

Meulenberg JJ, Petersen den Besten A, de Kluyver E, van Nieuwstadt A, Wensvoort G, Moormann RJ (1997) Molecular characterization of Lelystad virus. Vet Microbiol 55:197-202.

Mieli L, Chenoufi N, Lamanda P, Charreyre C (2002) Evaluation of different commercial elisa kits for detecting antibodies against prrs virus in europe. In: Proceedings of the International Pig Veterinary Society Congress, p358

Miller LC & Fox JM (2004) Apoptosis and porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 102:131-42.

Mills KH (2004) Regulatory T cells: friend or foe in immunity to infection? Nat Rev Immunol 4:841-55.

Mills KH & McGuirk P (2004) Antigen-specific regulatory T cells--their induction and role in infection. Semin Immunol 16:107-17.

Moestrup SK, & Moller HJ (2004) CD163: a regulated haemoglobin scavenger receptor with a role in the anti-inflammatory response. Ann Med 36:347-54.

Molitor TW, Bautista EM, Choi CS (1997) Immunity to PRRSV: double-edged sword. Vet Microbiol 55:265-76.

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19:683-765.

Mortensen S, Stryhn H, Sogaard R, Boklund A, Stark KD, Christensen J, Willeberg P (2002) Risk factors for infection of sow herds with porcine reproductive and respiratory syndrome (PRRS) virus. Prev Vet Med 53:83-101.

Murray PJ (1999) Defining the requirements for immunological control of mycobacterial infections. Trends Microbiol 7:366-72.

Murtaugh MP, Elam MR, Kakach LT (1995) Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. Arch Virol 140:1451-60.

Murtaugh MP, Yuan S, Faaberg KS (2001) Appearance of novel PRRSV isolates by recombination in the natural environment. Adv Exp Med Biol 494:31-6.

Murtaugh MP & Foss DI (2002) Inflammatory cytokines and antigen presenting cell activation. Vet Immunol Immunopathol 87:109-21.

Murtaugh MP, Xiao Z, Zuckermann F (2002) Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. Viral Immunol 15:533-47.

Nelsen CJ, Murtaugh MP, Faaberg KS (1999) Porcine reproductive and respiratory syndrome virus comparison: Divergent evolution on two continents. J Virol 73:270-80.

Nelson EA, Christopher-Hennings J, Benfield, DA (1994) Serum immune responses to the proteins of porcine reproductive and respiratory syndrome (PRRS) virus. J Vet Diagn Invest 6:410-5.

Neumann EJ, Kliebenstein JB, Johnson CD, Mabry JW, Bush EJ, Seitzinger AH, Green AL, Zimmerman JJ (2005) Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. J Am Vet Med Assoc 227:385-92.

Nielsen J & Botner A (1997) Hematological and immunological parameters of 4 1/2-month old pigs infected with PRRS. Vet Microbiol 55:289–94.

Nielsen TL, Nielsen J, Have P, Baekbo P, Hoff-Jorgensen R, Botner A (1997) Examination of virus shedding in semen from vaccinated and from previously infected boars after experimental challenge with porcine reproductive and respiratory syndrome virus. Vet Microbiol 54:101-12.

Nielsen HS, Oleksiewicz MB, Forsberg R, Stadejek T, Botner A, Storgaard T (2001) Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. J Gen Virol 82:1263-72.

Nielsen J, Botner A, Bille-Hansen V, Oleksiewicz MB, Storgaard T (2002) Experimental inoculation of late term pregnant sows with a field isolate of porcine reproductive and respiratory syndrome vaccine-derived virus. Vet Microbiol 84:1-13.

Nilubol D, Platt KB, Halbur PG, Torremorell M, Harris DL (2004) The effect of a killed porcine reproductive and respiratory syndrome virus (PRRSV) vaccine treatment on virus shedding in previously PRRSV infected pigs. Vet Microbiol 102:11-8.

Nodelijk G, van Leengoed LA, Schoevers EJ, Kroese AH, De Jong MC, Wensvoort G, Verheijden JH (1997) Seroprevalence of porcine reproductive and respiratory syndrome virus in Dutch weaning pigs. Vet Microbiol 56:21-32.

Nodelijk G, de Jong MC, Van Nes A, Vernooy JC, Van Leengoed LA, Pol JM, Verheijden JH (2000) Introduction, persistence and fade-out of porcine reproductive and respiratory syndrome virus in a Dutch breeding herd: a mathematical analysis. Epidemiol Infect 124:173-82.

Nodelijk G, Nielen M, De Jong MC, Verheijden JH (2003) A review of porcine reproductive and respiratory syndrome virus in Dutch breeding herds: population dynamics and clinical relevance. Prev Vet Med 60:37-52.

O'Connor M, Fallon M, O'Reylli PJ (2002) Detection of antibody to porcine reproductive and respiratory syndrome (PRRS) virus: Reduction of cut-off value of an ELISA, with confirmation by immunoperoxidase monolayers assay. Irish Vet J 55:73-5.

Ohlinger VF, Pesch S, Bischoff C (2000) History, occurrence, dynamics, and current status of PRRS in Europe. Vet Res 31:86-7.

Oleksiewicz MB, Botner A, Normann P (2001) Semen from boars infected with porcine reproductive and respiratory syndrome virus (PRRSV) contains antibodies against structural as well as nonstructural viral proteins. Vet Microbiol 81:109-25.

Olin MR, Batista L, Xiao Z, Dee SA, Murtaugh MP, Pijoan CC, Molitor TW (2005) Gammadelta lymphocyte response to porcine reproductive and respiratory syndrome virus. Viral Immunol 18:490-9.

Onyekaba CO, Harty JT, Even C, Hu BG, Plagemann PG (1989) Persistent infection of mice by lactate dehydrogenase-elevating virus: effects of immunosuppression on virus replication and antiviral immune responses. Virus Res 14:297-315.

Opriessnig T, Halbur PG, Yoon KJ, Pogranichniy RM, Harmon KM, Evans R, Key KF, Pallares FJ, Thomas P, Meng XJ (2002) Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. J Virol 76:11837-44.

Osorio FA, Galeota JA, Nelson E, Brodersen B, Doster A, Wills R, Zuckermann F, Laegreid WW (2002) Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. Virology 302:9-20.

Osorio FA & López OJ (2004) Qué factores o mecanismos protegen contra la infección por PRRSV. Importancia de la inmunidad humoral. En: Resúmenes de la XXXVII Semana Nacional del Ganado Porcino, Lorca, p55-66.

Ostrowski M, Galeota JA, Jar AM, Platt KB, Osorio FA, Lopez OJ (2002) Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J Virol 76:4241-50.

Otake S, Dee SA, Rossow KD, Joo HS, Deen J, Molitor TW, Pijoan C (2002a) Transmission of porcine reproductive and respiratory syndrome virus by needles. Vet Rec 150:114-115.

Otake S, Dee SA, Rossow KD, Moon RD, Pijoan C (2002b) Mechanical transmission of porcine reproductive and respiratory syndrome virus by mosquitoes, *Aedes vexans* (Meigen). Can J Vet Res 66:191-5.

Otake S, Dee SA, Rossow KD, Moon RD, Trincado C, Pijoan C (2003) Evaluation of mechanical transmission of porcine reproductive and respiratory syndrome virus by houseflies, *Musca domestica* (Linnaeus). Vet Rec 152:73-6.

Park BK, Yoon IJ, Joo HS (1996) Pathogenesis of plaque variants of porcine reproductive and respiratory syndrome virus in pregnant sows. Am J Vet Res 57:320-3.

Paton DJ, Brown IH, Scott AC, Done SH, Edwards S (1992) Isolation of a Lelystad virus-like agent from British pigs and scanning electron microscopy of infected macrophages. Vet Microbiol 33:195–201.

Pesch S, Meyer C, Ohlinger VF (2005) New insights into the genetic diversity of European porcine reproductive and respiratory syndrome virus (PRRSV). Vet Microbiol 107:31-48.

Philippidis P, Mason JC, Evans BJ, Nadra I, Taylor KM, Haskard DO, Landis RC (2004) Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxygenase-1 synthesis: anti-inflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. Circ Res 94:119-26.

Piras F, Bollard S, Laval F, Joisel F, Reynaud G, Charreyre C, Andreoni C, Juillard V (2005) Porcine reproductive and respiratory syndrome (PRRS) virus-specific interferon-gamma(+) T-cell responses after PRRS virus infection or vaccination with an inactivated PRRS vaccine. Viral Immunol 18:381-9.

Pirtle E & Beran G (1996) Stability of porcine reproductive and respiratory syndrome virus in the presence of fomites commonly found on farms. J Am Vet Med Assoc 08:390-2.

Pirzadeh B & Dea S (1997) Monoclonal antibodies to the ORF5 product of porcine reproductive and respiratory syndrome virus define linear neutralizing determinants. J Gen Virol 78:1867-73.

Pirzadeh B & Dea S (1998) Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus. J Gen Virol 79:989-99.

Pirzadeh B, Gagnon CA, Dea S (1998) Genomic and antigenic variations of porcine reproductive and respiratory syndrome virus major envelope GP5 glycoprotein. Can J Vet Res 62:170-7.

Plagemann PG & Moennig V (1992) Lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus: a new group of positive-strand RNA viruses. Adv Virus Res 41:99-192.

Plagemann PG (1996) Lactate dehydrogenase–elevating virus and related viruses. In: Fields Virology, 3d ed. Ed. Fields BN, Knipe DM, Howley PM, et al. Philadelphia, p1105-20.

Plagemann PG (2003) Porcine reproductive and respiratory virus: origin hypothesis. Emerg Infect Dis 9:903-5.

Plagemann PG (2004) The primary GP5 neutralization epitope of North American isolates of porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 102:263-75.

Plana-Duran J, Vayreda M, Vilarrasa J, Bastons M, Rosell R, Martinez M, San Gabriel A, Pujols J, Badiola JL, Ramos JA, et al. (1992a) Porcine epidemic abortion and respiratory syndrome (mystery swine disease). Isolation in Spain of the causative agent and experimental reproduction of the disease. Vet Microbiol 33:203-11.

Plana-Duran J, Vayreda M, Vilarrasa J, Bastons M, Rosell R, Martínez M, Pujols J, Badiola JI, Ramos JA, Domingo M, et al. (1992b) Síndrome reproductivo y respiratorio porcino (SRRP) o enfermedad misteriosa del cerdo (MPD): resultados preliminares del aislamiento del agente causal y reproducción experimental de la enfermedad. Anaporc 108:33-42.

Plana-Duran J, Bastons M, Urniza A, Vayreda M, Vila X, Mane H (1997a) Efficacy of an inactivated vaccine for prevention of reproductive failure induced by porcine reproductive and respiratory syndrome virus. Vet Microbiol 55:361-70.

Plana-Duran J, Climent I, Sarraseca J, Urniza A, Cortes E, Vela C, Casal JI (1997b) Baculovirus expression of proteins of porcine reproductive and respiratory syndrome virus strain Olot/91. Involvement of ORF3 and ORF5 proteins in protection. Virus Genes 14:19-29.

Polson DD, Marsh WE, Dial GD, Christianson WT (1992) Financial impact of porcine epidemic abortion and respiratory syndrome (PEARS). In: Proceedings of the 12th International Pig Veterinary Society Congress, p132.

Prieto C, Sanchez R, Martin-Rillo S, Suarez P, Simarro I, Solana A, Castro JM (1996) Exposure of gilts in early gestation to porcine reproductive and respiratory syndrome virus. Vet Rec 138:536-9.

Prieto C, Suarez P, Simarro I, Garcia C, Fernandez A, Castro JM (1997) Transplacental infection following exposure of gilts to porcine reproductive and respiratory syndrome virus at the onset of gestation. Vet Microbiol 57:301-11.

Prieto C, Garcia C, Simarro I, Castro JM (2003) Temporal localization of porcine reproductive and respiratory syndrome virus in reproductive tissues of experimentally infected boars. Theriogenology 60:1505-14.

Prieto C & Castro JM (2005) Porcine reproductive and respiratory syndrome virus infection in the boar: a review. Theriogenology 63:1-16.

Redpath S, Ghazal P, Gascoigne NR (2001) Hijacking and exploitation of IL-10 by intracellular pathogens. Trends Microbiol 9:86-92.

Reynaud G, Zimmerman M, Charreyre C, Brun A (1998) Field evaluation of an inactivated vaccine against PRRS. In: Proceedings of the 15th International Pig Veterinary Society Congress, p301.

Roberts J (2001) Applying PRRSV sequencing to field cases. Proc Allen D Leman Swine Conf 28:75-8.

Roberts J (2003) Monitoring infected sows herds using genomic sequencing. In: PRRS Compendium, 2d edition. Ed: Zimmerman JJ, Yoon KJ. Iowa, p.103-16.

Robertson IB (1992) Transmission of blue-eared pig disease. Vet Rec 130:478-9.

Roof MB, Gorcyca D, Wensvoort D (2000) Efficacy of a modified live porcine reproductive and respiratory syndrome virus vaccine (Ingelvac®PRRS MLV) against heterologous virulent Lelystad challenge. In: Proceedings of the 16th International Pig Veterinary Society Congress, p641.

Ropp SL, Wees CE, Fang Y, Nelson EA, Rossow KD, Bien M, Arndt B, Preszler S, Steen P, Christopher-Hennings J, Collins JE, Benfield DA, Faaberg KS (2004) Characterization of emerging European-like porcine reproductive and respiratory syndrome virus isolates in the United States. J Virol 78:3684-703.

Rossow KD, Bautista EM, Goyal SM, Molitor TW, Murtaugh MP, Morrison RB, Benfield DA, Collins JE (1994) Experimental porcine reproductive and respiratory syndrome virus infection in one-, four-, and 10-week-old pigs. J Vet Diagn Invest 6:3-12.

Rossow KD, Collins JE, Goyal SM, Nelson EA, Christopher-Hennings J, Benfield DA (1995) Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. Vet Pathol 32:361-73.

Rossow KD (1996) Pathogenesis of Porcine Reproductive and Respiratory Syndrome Virus. Ph.D. diss., Univ Minnesota, Minneapolis-St. Paul.

Rossow KD, Benfield DA, Goyal SM, Nelson EA, Christopher-Hennings J, Collins JE (1996) Chronological immunohistochemical detection and localization of porcine reproductive and respiratory syndrome virus in gnotobiotic pigs. Vet Pathol 33:551-6.

Rossow K, Vaughn E, Nelson E (1997) Investigation of an abortion storm in a RespPRRS® vaccinated swine herd from SE Iowa. In: Proceedings of the Allen D. Leman Swine Conference (Research Abstracts), p1.

Rossow KD (1998) Porcine reproductive and respiratory syndrome. Vet Pathol 35:1-20.

Rossow KD, Shivers JL, Yeske PE (1999) Porcine reproductive and respiratory syndrome virus infection in neonatal pigs characterized by marked neurovirulence. Vet Rec 144:444-8.

Rousset F, Garcia E, Defrance T, Peronne C, Vezzio N, Hsu DH, Kastelein R, Moore KW, Banchereau J (1992) Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc Natl Acad Sci USA 89:1890-3.

Rowbottom AW, Lepper MA, Garland RJ, Cox CV, Corley EG (1999) Interleukin-10-induced CD8 cell proliferation. Immunology 98:80-9.

Rowland RR, Steffen M, Ackerman T, Benfield D (1999) The evolution of porcine reproductive and respiratory syndrome virus: quasispecies and emergence of a virus subpopulation during infection of pigs with VR-2332. Virology 259:262-6.

Rowland RR, Robinson B, Stefanick J, Kim TS, Guanghua L, Lawson SR, Benfield DA (2001) Inhibition of porcine reproductive and respiratory syndrome virus by interferon-gamma and recovery of virus replication with 2-aminopurine. Arch Virol 146:539-55.

Royaee AR, Husmann RJ, Dawson HD, Calzada-Nova G, Schnitzlein WM, Zuckermann FA, Lunney JK (2004) Deciphering the involvement of innate immune factors in the development of the host response to PRRSV vaccination. Vet Immunol Immunopathol 102:199-216.

Samsom JN, de Bruin TG, Voermans JJ, Meulenberg JJ, Pol JM, Bianchi AT (2000) Changes of leukocyte phenotype and function in the broncho-alveolar lavage fluid of pigs infected with porcine reproductive and respiratory syndrome virus: a role for CD8(+) cells. J Gen Virol 81:497-505.

Schols D & De Clercq E (1996) Human immunodeficiency virus type 1 gp120 induces anergy in human peripheral blood lymphocytes by inducing interleukin-10 production. J Virol 70:4953-60.

Scortti M, Prieto C, Martinez-Lobo FJ, Simarro I, Castro JM (en prensa) Effects of two commercial European modified-live vaccines against porcine reproductive and respiratory syndrome viruses in pregnant gilts. Vet J.

Shen S, Kwang J, Liu W, Liu DX (2000) Determination of the complete nucleotide sequence of a vaccine strain of porcine reproductive and respiratory syndrome virus and identification of the Nsp2 gene with a unique insertion. Arch Virol 145:871-83.

Shibata I, Mori M, Uruno K, Samegai Y, Okado M (1997) In vivo replication of porcine reproductive and respiratory syndrome virus in swine alveolar macrophages and change in the cell population in bronchoalveolar lavage fluid after infection. J Vet Med Sci 59:539–43.

Shibata I, Mori M, Uruno K (1998) Experimental infection of maternally immune pigs with porcine reproductive and respiratory syndrome (PRRS) virus. J Vet Med Sci 60:1285-91.

Shimizu M, Yamada S, Kawashima K, Ohashi S, Shimizu S, Ogawa T (1996) Changes of lymphocyte subpopulations in pigs infected with porcine reproductive and respiratory syndrome (PRRS) virus. Vet Immunol Immunopathol 50:19-27.

Shin JH, Kang YB, Kim YJ (1993) Seroepidemiological studies on porcine reproductive and respiratory syndrome in Korea. I. Detection of indirect fluorescent antibodies. RDA J Agri Sci 35:572-6.

Shin JH, Torrison J, Choi CS, Gonzalez SM, Crabo BG, Molitor TW (1997) Monitoring of porcine reproductive and respiratory syndrome virus infection in boars. Vet Microbiol 55:337-46.

Sipos W, Duvigneau C, Pietschmann P, Holler K, Hartl R, Wahl K, Steinborn R, Gemeiner M, Willheim M, Schmoll F (2003) Parameters of humoral and cellular immunity following vaccination of pigs with a European modified-live strain of porcine reproductive and respiratory syndrome virus (PRRSV). Viral Immunol. 16:335-46.

Sirinarumitr T, Zhang Y, Kluge JP, Halbur PG, Paul PS (1998) A pneumo-virulent United States isolate of porcine reproductive and respiratory syndrome virus induces apoptosis in bystander cells both in vitro and in vivo. J Gen Virol 79:2989-95.

Snijder EJ, Meulenberg JJ (1998) The molecular biology of arteriviruses. J Gen Virol 79: 961–79.

Storgaard T, Oleksiewicz M, Bøtner A (1999) Examination of the selective pressures on a live PRRS vaccine virus. Arch Virol 144:2389-401.

Suárez P, Diaz-Guerra M, Prieto C, Esteban M, Castro JM, Nieto A, Ortin J (1996) Open reading frame 5 of porcine reproductive and respiratory syndrome virus as a cause of virus-induced apoptosis. J Virol 70:2876-82.

Sundstedt A, Hoiden I, Rosendahl A, Kalland T, van Rooijen N, Dohlsten M (1997) Immunoregulatory role of IL-10 during superantigen-induced hyporesponsiveness in vivo. J Immunol 158:180-6.

Sur JH, Cooper VL, Galeota JA, Hesse RA, Doster AR, Osorio FA (1996) In vivo detection PRRS virus RNA by in situ hybridization at different times postinfection. J Clin Microbiol 34:2280-6.

Sur JH, Doster AR, Christian JS, Galeota JA, Wills RW, Zimmerman JJ, Osorio FA (1997) Porcine reproductive and respiratory syndrome virus replicates in testicular germ cells, alters spermatogenesis and induces germ cell death by apoptosis. J Virol 71:9170–9.

Sur JH, Doster AR, Osorio FA (1998) Apoptosis induced in vivo during acute infection by porcine reproductive and respiratory syndrome virus. Vet Pathol 35:506-14.

Sur JH, Doster AR, Galeota JA, Osorio FA (2001) Evidence for the localization of porcine reproductive and respiratory syndrome virus (PRRSV) antigen and RNA in ovarian follicles in gilts. Vet Pathol 38:58-66.

Suradhat S & Thanawongnuwech R (2003) Upregulation of interleukin-10 gene expression in the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus. J Gen Virol 84:2755-60.

Suradhat S, Thanawongnuwech R, Poovorawan Y (2003) Upregulation of IL-10 gene expression in porcine peripheral blood mononuclear cells by porcine reproductive and respiratory syndrome virus. J Gen Virol 84:453-9.

Swenson SL, Hill HT, Zimmerman JJ, Evans LE, Landgraf JG, Wills RW, Sanderson TP, McGinley MJ, Brevik AK, Ciszewski DK, Frey ML (1994) Excretion of porcine reproductive and respiratory syndrome virus in semen after experimentally induced infection in boars. J Am Vet Med Assoc 204:1943-8.

Swenson SL, Hill HT, Zimmerman JJ, Evans LE, Wills RW, Yoon KJ, Schwartz KJ, Althouse GC, McGinley MJ, Brevik AK (1995) Preliminary assessment of an inactivated PRRS virus vaccine on the excretion of virus in semen. Swine Health and Production 3:244-47.

Takikawa N, Kobayashi S, Ide S, Yamane Y, Tanaka Y, Higashihara M, Yamagishi H (1997) Early serodiagnosis of porcine reproductive and respiratory syndrome virus infection of pigs by detection of slow-reacting and complement-requiring neutralizing antibody. J Vet Med Sci ;59:31-4.

Terpstra C, Wensvoort G, Pol JM (1991) Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. Vet Q 13:131-6.

Terpstra C, Wensvoort G, Van Leengoed LAMG (1992) Persistence of Lelystad virus in herds affected by porcine epidemic abortion and respiratory syndrome. In: Proceedings of the 12th International Pig Veterinary Society Congress, p118.

Thanawongnuwech R, Thacker EL, Halbur PG (1998) Influence of pig age on virus titer and bactericidal activity of porcine reproductive and respiratory syndrome virus (PRRSV)-infected pulmonary intravascular macrophages (PIMs). Vet Microbiol 63:177-87.

Thanawongnuwech R, Halbur PG, Thacker EL (2000) The role of pulmonary intravascular macrophages in porcine reproductive and respiratory syndrome virus infection. Anim Health Res Rev 1:95-102.

Thanawongnuwech R, Young TF, Thacker BJ, Thacker EL (2001) Differential production of proinflammatory cytokines: in vitro PRRSV and Mycoplasma hyopneumoniae co-infection model. Vet Immunol Immunopathol 79:115-27.

Thanawongnuwech R, Amonsin A, Tatsanakit A, Damrongwatanapokin S (2004) Genetics and geographical variation of porcine reproductive and respiratory syndrome virus (PRRSV) in Thailand. Vet Microbiol 101:9-21.

Tingstedt JE & Nielsen J (2004) Cellular immune responses in the lungs of pigs infected in utero with PRRSV: an immunohistochemical study. Viral Immunol 17:558-64.

Torremorell M, Pijoan C, Janni K, Walker R, Joo HS (1997) Airborne transmission of Actinobacillus pleuropneumoniae and porcine reproductive and respiratory syndrome virus in nursery pigs. Am J Vet Res 58:828-32.

Van Alstine WG, Popielarczyk M, Albregts SR (2002) Effect of formalin fixation on the immunohistochemical detection of PRRS virus antigen in experimentally and naturally infected pigs. J Vet Diagn Invest 14:504-7.

Van den Broek MF, Sporri R, Even C, Plagemann PG, Hanseler E, Hengartner H, Zinkernagel RM (1997) Lactate dehydrogenase-elevating virus (LDV): lifelong coexistence of virus and LDV-specific immunity. J Immunol 159:1585-8.

Vanderheijden N, Delputte PL, Favoreel HW, Vandekerckhove J, Van Damme J, van Woensel PA, Nauwynck HJ (2003) Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. J Virol 77:8207-15.

Van der Linden IFA, Voermans JJM, Vand der Linde-Bril EM, Bianchi ATJ, Steverink PJGM (2003) Virological kinetics and immunological response to a porcine reproductive and respiratory syndrome virus infection of pigs at different ages. Vaccine 21:1952-7.

Van Nieuwstadt AP, Meulenberg JJ, van Essen-Zanbergen A, Petersen-den Besten A, Bende RJ, Moormann RJ, Wensvoort G (1996) Proteins encoded by open reading frames 3 and 4 of the genome of Lelystad virus (Arteriviridae) are structural proteins of the virion. J Virol 70:4767-72.

Van Reeth K, Labarque G, Nauwynck H, Pensaert M (1999) Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. Res Vet Sci 67:47-52.

Van Reeth K, Van Gucht S, Pensaert M (2002) In vivo studies on cytokine involvement during acute viral respiratory disease of swine: troublesome but rewarding. Vet Immunol Immunopathol 87:161-8.

Van Vugt JJ, Storgaard T, Oleksiewicz MB, Botner A (2001) High frequency RNA recombination in porcine reproductive and respiratory syndrome virus occurs preferentially between parental sequences with high similarity. J Gen Virol 82:2615-20.

Van Woensel PA, Liefkens K, Demaret S (1998) European serotype PRRSV vaccine protects against European serotype challenge whereas an American serotype vaccine does not. Adv Exp Med Biol 440:713-8.

Verheije MH, Olsthoorn RC, Kroese MV, Rottier PJ, Meulenberg JJ (2002) Kissing interaction between 3' noncoding and coding sequences is essential for porcine arterivirus RNA replication. J Virol 76:1521-6.

Vezina, SA, Loemba H, Fournier M, Dea S, Archambault D (1996) Antibody production and blastogenic response in pigs experimentally infected with porcine reproductive and respiratory syndrome virus. Can J Vet Res 60:94-9.

Vincent AL, Thacker BJ, Halbur PG, Rothschild MF, Thacker EL (2005) In vitro susceptibility of macrophages to porcine reproductive and respiratory syndrome virus varies between genetically diverse lines of pigs. Viral Immunol 18:506-12.

Vincent AL, Thacker BJ, Halbur PG, Rothschild MF, Thacker EL (2006) An investigation of susceptibility to porcine reproductive and respiratory syndrome virus between two genetically diverse commercial lines of pigs. J Anim Sci 84:49-57.

Voicu IL, Silim A, Morin M, Elazhary MA (1994) Interaction of porcine reproductive and respiratory syndrome virus with swine monocytes. Vet Rec 134:422-3.

Wagstrom EA, Chang C-C, Yoon K-J, Zimmerman JJ (2001) Shedding of porcine reproductive and respiratory syndrome (PRRS) virus in mammary secretions of sows. Am J Vet Res 62:1876-80.

Wasilk A, Callahan JD, Christopher-Hennings J, Gay TA, Fang Y, Dammen M, Reos ME, Torremorell M, Polson D, Mellencamp M, Nelson E, Nelson WM (2004) Detection of U.S., Lelystad, and European-like porcine reproductive and respiratory syndrome viruses and relative quantitation in boar semen and serum samples by real-time PCR. J Clin Microbiol 42:4453-61.

Wee GJ, Cao MJ, Liu W, Kwang J (2001) Efficacy of porcine reproductive and respiratory syndrome virus vaccine and porcine interleukin-12. Vet. Therapeutics 2:112–9.

Weiland E, Wieczorek-Krohmer M, Kohl D, Conzelmann KK, Weiland F (1999) Monoclonal antibodies to the GP5 of porcine reproductive and respiratory syndrome virus are more effective in virus neutralization than monoclonal antibodies to the GP4. Vet Microbiol 66:171-86.

Wensvoort G, Terpstra C, Pol JM, ter Laak EA, Bloemraad M, de Kluyver EP, Kragten C, van Buiten L, den Besten A, Wagenaar F, et al. (1991) Mystery swine disease in the Netherlands: the isolation of Lelystad virus. Vet Q 13:121-30.

Westermann J & Pabst R (1992) Distribution of lymphocyte subsets and natural killer cells in the human body. Clin Investig 70:539-44.

White MEC (1991) Blue ear disease of pigs. Vet Rec 128:574.

White MEC (1992) The clinical signs and symptoms of "blue eared pig disease." Pig Vet J 28:62-8.

Wills R, Zimmerman J, Yoon KJ, Swenson S, McGinley M, Hill H, Platt K, Christopher-Hennings J, Nelson E (1995) Porcine reproductive and respiratory syndrome virus: A persistent infection. In: Proceedings of the 2d International Symposium on Porcine Reproductive and Respiratory Syndrome, p19.

Wills RW, Zimmerman JJ, Yoon KJ, Swenson SL, Hoffman LJ, McGinley MJ, Hill HT, Platt KB (1997a) Porcine reproductive and respiratory syndrome virus: Routes of excretion. Vet Microbiol 57:69-81.

Wills RW, Zimmerman JJ, Swenson SL, Yoon K-J, Hill HT, Bundy DS, McGinley MJ (1997b) Transmission of porcine reproductive and respiratory syndrome virus by direct close or indirect contact. Swine Health and Production 5:213-8.

Wills RW, Zimmerman JJ, Yoon KJ, Swenson SL, McGinley MJ, Hill HT, Platt KB, Christopher-Hennings J, Nelson EA (1997c) Porcine reproductive and respiratory syndrome virus: A persistent infection. Vet Microbiol 55:231-40.

Wills RW, Doster AR, Galeota JA, Sur JH, Osorio FA (2003) Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. J Clin Microbiol 41:58-62.

Wissink EH, van Wijk HA, Kroese MV, Weiland E, Meulenberg JJ, Rottier PJ, van Rijn PA (2003) The major envelope protein, GP5, of a European porcine reproductive and respiratory syndrome virus contains a neutralization epitope in its N-terminal ectodomain. J Gen Virol 84:1535-43.

Wootton S, Yoo D, Rogan D (2000) Full-length sequence of a Canadian porcine reproductive and respiratory syndrome virus (PRRSV) isolate. Arch Virol 145:2297-323.

Wu WH, Fang Y, Farwell R, Steffen-Bien M, Rowland RR, Christopher-Hennings J, Nelson EA (2001) A 10-kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF 2b. Virology 287:183-91.

Wu WH, Fang Y, Rowland RR, Lawson SR, Christopher-Hennings J, Yoon KJ, Nelson EA (2005) The 2b protein as a minor structural component of PRRSV. Virus Res 114:177-81.

Xiao Z, Batista L, Dee S, Halbur P, Murtaugh MP (2004) The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. J Virol 78:5923-33.

Xue Q, Zhao YG, Zhou YJ, Qiu HJ, Wang YF, Wu DL, Tian ZJ, Tong GZ (2004) Immune responses of swine following DNA immunization with plasmids encoding porcine reproductive and respiratory syndrome virus ORFs 5 and 7, and porcine IL-2 and IFNgamma. Vet Immunol Immunopathol 102:291-8.

Yaeger MJ, Prieve T, Collins J, Christopher-Hennings J, Nelson E., Benfield D (1993) Evidence for the transmission of porcine reproductive and respiratory syndrome (PRRS) virus in boar semen. Swine Health and Production 1:7-9.

Yang L, Frey ML, Yoon KJ, Zimmerman JJ, Platt KB (2000) Categorization of North American porcine reproductive and respiratory syndrome viruses: epitopic profiles of the N, M, GP5 and GP3 proteins and susceptibility to neutralization. Arch Virol 145:1599-619.

Yoon IJ, Joo HS, Goyal SM, Molitor TW (1994) A modified serum neutralization test for the detection of antibody to porcine reproductive and respiratory syndrome virus in swine sera. J Vet Diagn Invest 6:289-92.

Yoon KJ, Zimmerman JJ, Swenson SL, McGinley MJ, Eernisse KA, Brevik A, Rhinehart LL, Frey ML, Hill HT, Platt KB (1995) Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. J Vet Diagn Invest 7:305-12.

Yoon KJ, Wu LL, Zimmerman JJ, Platt KB (1996) Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. Virol Immunol 9:51-63.

Yoon KJ, Wu LL, Zimmerman JJ, Platt KB (1997) Field isolates of porcine reproductive and respiratory syndrome virus (PRRSV) vary in their susceptibility to antibody dependent enhancement (ADE) of infection. Vet Microbiol 55:277-87.

Yoon KJ, Christopher-Hennings J, Nelson EA (2003) Diagnosis. In: PRRS Compendium, 2d edition. Ed: Zimmerman JJ, Yoon KJ. Iowa, p.59-74.

Yoshii M, Kaku Y, Murakami Y, Shimizu M, Kato K, Ikeda H (2005) Genetic variation and geographic distribution of porcine reproductive and respiratory syndrome virus in Japan. Arch Virol 150:2313-24.

Yuan S, Mickelson D, Murtaugh MP, Faaberg KS (2001) Complete genome comparison of porcine reproductive and respiratory syndrome virus parental and attenuated strains. Virus Res 74:99-110.

Zhou EM, Zimmerman JJ, Zhou KX, Jiang Z, Platt KB (2002) IgM-capture ELISA as a serodiagnostic test for detection of primary infection of PRRSV in pigs. Proc Annu Meet Am Assoc Swine Vet, p295-8.

Zimmerman JJ, Yoon KJ, Pirtle EC, Wills RW, Sanderson TJ, McGinley MJ (1997) Studies of porcine reproductive and respiratory syndrome (PRRS) virus infection in avian species. Vet Microbiol 55:329-36.

Zimmerman JJ (2003a) Historical Overview of PRRS virus. In: PRRS Compendium, 2d edition. Ed: Zimmerman JJ, Yoon KJ. Iowa, p1-6.

Zimmerman JJ (2003b) Epidemiology and Ecology. In: PRRS Compendium, 2d edition. Ed: Zimmerman JJ, Yoon KJ. Iowa, p27-50.

Zimmerman JJ, Benfield DA, Murtaugh P, Osorio F, Stevenson GW, Torremorell M (2006) Porcine Reproductive and Respiratory Syndrome Virus (Porcine Arterivirus). In: Diseases of Swine, 9th ed. Ed: Straw BA, Zimmerman JJ, D'Allaire S, Taylor DJ. Iowa, p387-416.

Zuckermann FA, Husmann RJ, Schwartz R, Brandt J, Mateu E, Martin S (1998) Interleukin-12 enhances the virus-specific interferon gamma responses of pigs to an inactivated pseudorabies virus vaccine. Vet Immunol Immunopathol **63**:57–67.

Zuckermann FA, Martin S, Husmann RJ, Brandt J (1999) Use of interleukin 12 to enhance the cellular immune response of swine to an inactivated herpesvirus vaccine. Adv Vet Med 41:447-61.