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# Participación de los linfocitos CD4+CD8- y CD4+CD8+ durante la respuesta inmune al rubulavirus porcino

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## INDICE GENERAL

**Resumen**

Este trabajo describe los principales mecanismos generados en la respuesta inmune al rubulavirus porcino y propone nuevas alternativas para estudiar los mecanismos involucrados en la memoria inmunológica del cerdo. Los resultados muestran que se detectaron anticuerpos específicos a la proteína HN del virus a partir de la primera semana de infección. Las células mononucleares (CMN) proliferaron *in vitro* en presencia de virus a partir de la segunda semana postinfección ($p<0.05$), y el análisis del fenotipo mostró un incremento en CD4+CD8- y CD4+CD8+ ($p<0.05$). Las CMN estimuladas con fitohemaglutinina (PHA) sólo incrementaron el fenotipo CD4+CD8-. En la primera semana postinfección aumentaron los valores de linfocitos CD4-CD8+ y en la tercera disminuyeron los CD4+CD8- ($p<0.05$). Este trabajo es el primero en describir el incremento *in vivo* en los porcentajes de linfocitos CD4+CD8+ después de una infección ($p<0.05$).

Los linfocitos CD4+CD8- participaron en las primeras semanas postinfección en la respuesta al virus y generaron linfocitos CD4+CD8+. Este fenómeno no se observó en linfocitos estimulados con PHA. Los linfocitos CD4+CD8+ participaron en la respuesta de memoria al virus y no modificaron su fenotipo después del estimuló con virus o PHA. El perfil de citocinas reveló que los linfocitos CD4+CD8+ expresaron citocinas de una célula de memoria con capacidad de regular el sistema inmune, mientras que los CD4+CD8- produjeron citocinas de una célula virgen. La lectina de cacahuate (PNA) y de amaranto (ALL) reconocieron un mayor porcentaje de linfocitos CD4+CD8+ ($p<0.05$) que de linfocitos CD4+CD8-. Los linfocitos reconocidos por PNA presentaron un fenotipo de memoria y los reconocidos por ALL presentaron un fenotipo de célula virgen o en transición, esto utilizando simultáneamente el anticuerpo anti-CD29. Los linfocitos PNA+CD29+ incrementaron significativamente después de la infección ($p<0.05$), y las células ALL+CD29+ no ($p>0.05$). Finalmente se determinó que los linfocitos PNA+ proliferaron en presencia del virus ($p<0.05$) y las células ALL+ no ($p>0.05$). En este trabajo se plantea la participación del sistema inmune durante la infección experimental con el rubulavirus porcino.

**Abstract**

Immune response against the porcine rubulavirus was analyzed in experimentally infected adult pigs. High titers of virus neutralizing and hemagglutinating inhibitory antibodies were identified in infected animals. The antibody specificity was directed towards HN, M, and NP rubulavirion proteins and immunodominance to HN proteins was demonstrated. Peripheral blood mononuclear cells from infected, but not from non-infected pigs proliferated *in vitro* in response to virus-antigen stimuli, showing a bell-shaped plot with the highest peak at 5 week postinfection. Virus-induced lymphoblasts expressed CD4+CD8+ phenotype, whereas lectin-induced lymphoblasts were mainly identified as CD4+CD8- cells. Phenotype analysis of freshly prepared PBMC revealed increased number of both monocytes (PoM1+) and total T lymphocytes (CD2+) early during infection, with reduced values of B lymphocytes at 4 week postinfection. Decrease in $CD4^+CD8^-$ blood cells was observed at 3 week postinfection, whereas both $CD4^-CD8^+$ and $CD4^+CD8^+$ cells increased at 1 and 4 weeks postinfection, respectively. Indicating the relevance of $CD4^+CD8^+$ T cells in the control of porcine rubulavirus infection. This work also analyzes the biological function of CD4+CD8- and CD4+CD8+ lymphocytes in the immune response to the porcine rubulavirus. CD4+CD8- cells isolated from porcine rubulavirus infected pigs after three weeks of post-infection proliferated in response to homologous virus and generated lymphoblasts which were predominantly of the CD4+CD8+ phenotype; stimulation of CD4+CD8- cells with mitogen did not switch the phenotype. CD4+CD8- lymphocytes isolated after 10 weeks of infection proliferated in response to homologous virus and PHA but did not change their phenotype. CD4+CD8+ lymphocytes responded to PHA or viral antigen but did not change their phenotype The cytokine profile of both lymphocyte populations stimulated with PMA/Ionomicine showed the presence of IL-2 and IL-10 transcripts, but their quantitation demonstrated that double positive cells express mainly IL-10 whereas CD4+CD8- lymphocytes express basically IL-2. Our results show that CD4+CD8- lymphocytes participating in the early phase of the porcine rubulavirus infection, regulate memory phenotype expression.

## INTRODUCCION

### Antecedentes de la enfermedad del ojo azul del cerdo

El rubulavirus porcino es responsable de la enfermedad del ojo azul de los cerdos, que se caracteriza por ocasionar problemas neurológicos, respiratorios y reproductivos (1-3). Un 10% de los animales infectados presentan opacidad de la córnea, de ahí el nombre de la enfermedad (2). El rubulavirus porcino se clasifica dentro del género *Rubulavirus*, subfamilia *Paramixovirinae* y familia *Paramixoviridae* (4). Este es un virus ARN de cadena negativa, pleomórfico de 150 a 300 nm, envuelto y con proyecciones en su membrana comunes en los paramixovirus (5, 6). Este virus aglutina eritrocitos de diferentes especies animales incluyendo los humanos y posee actividad de neuraminidasa (2, 5). El virus se multiplica en una amplia variedad de líneas celulares, en las cuales forma sincicios, células vacuoladas, provoca la muerte celular (2, 5) y tiene la capacidad de establecer infecciones persistentes en células PK-15 (7). El patrón electroforético en geles de poliacrilamida bajo condiciones reductoras es similar al de otros paramixovirus, pero estudios serológicos no demuestran relación alguna con estos virus (3, 5, 8). Sin embargo, en nuestro laboratorio hemos observado que existe reacción cruzada entre sueros de cerdos infectados con el rubulavirus porcino y sueros de niños infectados con el virus de las paperas cuando son analizados por ELISA (9).

La caracterización de diferentes aislamientos del rubulavirus además de revelar que existen diferencias en su capacidad hemaglutinante, de neuraminidasa y en su patogenia (10, 11) ha llevado a la caracterización de una nueva cepa del virus. Esta cepa denominada originalmente Jalisco/1992 actualmente recibe el nombre PAC-3 (Producción Animal Cerdos). Este virus como los descritos originalmente, tiene la capacidad de multiplicarse en una amplia variedad de líneas celulares y aglutinar eritrocitos de diferentes especies animales; los estudios de microscopía revelan la estructura de un paramixovirus y es capaz de provocar alteraciones neurológicas en lechones y orquitis en adultos (12).

El virus está compuesto de seis proteínas básicamente (13). La proteína Mayor (L) de 200 KDa que posee actividad de RNA polimerasa dependiente de RNA; la Nucleoproteína (NP) de 68 kDa de función estructural que da origen junto con el RNA a la nucleocápside viral; la glicoproteína Hemaglutinina-Neuraminidasa (HN) de 66 kDa encargada del reconocimiento del celular; la proteína de Fusión (F) de 59 kDa responsable de la fusión de las membranas virales y celulares; la Fosfoproteína (P) de 52 kDa que participa en la regulación de la replicación y transcripción del genoma viral; y la proteína de Matriz (M) de 40 kDa que participa en el ensamblaje de los viriones (14).

Ensayos de competencia con diferentes azúcares demostraron que al igual que los paramixovirus, este virus reconoce ácido siálico. Sin embargo, su especificidad es mayor por el trisacárido sialil(α2,3)lactosa (ácido siálico α2,3Galß1,4Glc), ya que este azúcar inhibe las actividades hemaglutinante y formadora de sincicios en cultivos celulares (15). Estudios posteriores con inhibidores de la glicosilación y esteroides como la dexametasona, que favorecen la expresión en la membrana de sialil(α2,6)lactosa, en lugar de su isómero sialil(α2,3)lactosa, lograron bloquear la unión del virus al receptor celular y evitar así la infección. Estos resultados demuestran que la presencia del sialil(α2,3)lactosa es determinante para que el virus inicie su proceso de infección (16). La expresión tisular del sialil(α2,3)lactosa en cerdos sanos (17) ha revelado una estrecha relación con los sitios de replicación viral identificados en cerdos infectados natural y experimentalmente (18, 12). Los resultados obtenidos por inmunofluorescencia en cortes histológicos sugieren que la diferente patología del virus en cerdos lactantes y en cerdos adultos, obedece a la expresión diferencial de glicoconjugados con NeuAcα2,3Lac terminal, que se encuentra en mayor proporción en sistema nervioso de neonatos y en sistema reproductor de cerdos adultos, diferencias que pueden estar dirigidas por la actividad hormonal durante el proceso de maduración de los animales (17).

2, INF-γ y TNF-β y modulan la respuesta mediada por células. Los linfocitos cooperadores Th2 producen IL-4 e IL-10 y regulan la respuesta mediada por anticuerpos. La producción de uno u otro tipo de citocinas determina la resolución o no de la infección, y la elección de uno u otro tipo de respuesta está en función del tipo de célula presentadora, de las características del antígeno y del microambiente en el cual los antígenos son presentados a los linfocitos T (48). En la población de linfocitos CD4+CD8- porcinos no ha sido posible identificar esta dicotomía, pero existen evidencias que hacen suponer su existencia (42).

La población de linfocitos CD4-CD8+ se encarga de la citotoxicidad restringida por las moléculas del complejo principal de histocompatibilidad de clase-I (MHC-I), y es importante en las infecciones opr germenes intracelulares (49). Al igual que en los linfocitos CD4+CD8-, los linfocitos citotóxicos presenta una población Tc1 y otra Tc2 con el mismo patrón de citocinas (50). Diferentes estudios han demostrado la presencia de células citotóxicas en el cerdo, las cuales se encuentran restringidas por el MHC-I como en otras especies (51). La población citotóxica de cerdos ha mostrado ser CD5+CD6+CD8+ (52-54). Los linfocitos CD4+CD8+ en el cerdo varían de acuerdo a la edad del animal, y van desde el 1% en el primer mes hasta el 60% en animales mayores de 3 años (36). Los linfocitos CD4+CD8+ dobles positivos (DP), difieren de los linfocitos inmaduros DP en su tamaño y en la expresión de algunos marcadores de superficie como el CD1 (37) y en la molécula CD8 que en linfocitos inmaduros se expresa como heterodímeros α/β, mientras que en los linfocitos CD4+CD8+ se ha supuesto la presencia del homodímero α/α (38, 39). Los linfocitos CD4+CD8+ dobles positivos, se caracterizan por expresar la molécula CD3 (34) característica de los linfocitos T, moléculas del MHC (40) y la integrina α4/β4 o CD29 (36).

Algunas propiedades biológicas de los linfocitos CD4+CD8+ han demostrado que la respuesta proliferativa *in vitro* de esta población está restringida por el MHC-II (41), ya que los linfocitos CD4+CD8+ incubados con un anticuerpo anti-MHC-II y anti-CD4 disminuyen significativamente la respuesta proliferativa al virus de la enfermedad de Aujeszky. Células incubadas con un

anticuerpo anti-MHC-I o anti-CD8 no modifican la respuesta, lo que hace suponer que la actividad proliferativa de los linfocitos CD4+CD8+ está restringida por el MHC-II (41). En los cultivos de CMN se ha observado que la cantidad de linfocitos CD4+CD8+ se incrementa cuando se estimulan con antígenos como el virus de la enfermedad de Aujeszky (36, 37), el virus de la fiebre porcina africana (42), el virus de la viruela porcina (36), el rubulavirus porcino (43) y con algunos parásitos como *Trichinella spirales* (44). *In vitro,* se ha demostrado que esta población celular se puede generar a partir de los linfocitos CD4+CD8- tras el estímulo antigénico (36). *In vivo,* se ha observado que después de un proceso infeccioso estos linfocitos tienden a incrementarse (43). Estos resultados indican que la estimulación antigénica de los linfocitos CD4+CD8-, de un animal previamente sensibilizado, conduce a la adquisición del marcador CD8 y genera linfocitos CD4+CD8+. La participación de los linfocitos CD4+CD8- y CD4+CD8+ en la infección con el virus de la enfermedad de Aujeszky presenta resultados controvertidos (36, 41).

**La memoria inmunológica del cerdo**

Una vez que los linfocitos CD4 reconocen un antígeno presentado por las células dendríticas, proliferan y se diferencian en células efectoras o de memoria con capacidad para producir citocinas Th1 y/o Th2 (55). La mayoría de estas células mueren por la activación, y por la ausencia de citocinas y estímulo antigénico (56), sin embargo una fracción sobrevive como célula de memoria (57) siendo capaz de responder más rápido una vez que se encuentra con el mismo antígeno. Sin embargo, el contacto repetido con un antígeno conduce a una expansión rápida seguida por la muerte celular inducida por la activación y por lo tanto no favorece el desarrollo de la memoria (58-61). Con base en marcadores de superficie, los linfocitos T maduros se pueden distinguir entre linfocitos virgen y de memoria. Estas células presentan diferentes comportamientos *in vivo;* los linfocitos con fenotipo virgen persisten por largos periodos sin dividirse, mientras que los

linfocitos con fenotipo de memoria presentan células en movimiento capaces de activarse más rápidamente.

Los marcadores que caracterizan a los linfocitos de memoria incluyen el CD45R0, CD44, CD29, CD27 y el CD62 (62), así como la secreción de algunas citocinas tipo Th1 o Th2 (63). En el cerdo se han reportado anticuerpos contra los antígenos CD29 (36) y CD45R0 (64, 65) que se han relacionado con la memoria inmunológica en el humano y el ratón. De acuerdo con estos y otros resultados esta población celular se clasifica como una célula de memoria.

Los linfocitos CD4+CD8+ expresan la integrina $\alpha 4/\beta 4$ o CD29, que en los linfocitos humanos se ha relacionado con la memoria inmunológica (36). El CD29 en los linfocitos humanos y porcinos se presenta con altos niveles de expresión en los linfocitos efectores de memoria y en niveles bajos de expresión en los linfocitos vírgenes (36, 66). Se ha observado que 75% de los linfocitos porcinos CD4+CD8+ expresan altos niveles de CD29 ($CD29^{Alto}$), mientras que el 25% restante expresa bajos niveles ($CD29^{Bajo}$). Por el contrario, sólo el 25% de los linfocitos CD4+CD8- expresan altos niveles de CD29 y el 75% niveles bajos. Las diferencias en la expresión de la molécula CD29 coinciden con la capacidad de estas células para proliferar en presencia del virus. En este trabajo, los linfocitos $CD4+CD29^{Alto}$ presentaron una fuerte respuesta en presencia del virus, mientras que los linfocitos $CD4+CD29^{Bajo}$ presentaron una mínima respuesta. Por el contrario, los linfocitos $CD4+CD29^{Bajo}$ tuvieron una respuesta 5 veces mayor a PHA que los linfocitos $CD4+CD29^{Alto}$. Con base en estos resultados se propuso que los linfocitos CD4+CD8+ constituyen una subpoblación de células-efectoras de memoria (36).

Otros reportes que implican a los linfocitos CD4+CD8+ como células de memoria muestran que además estas células tienen la capacidad de cooperar con los linfocitos B para la producción de anticuerpos durante la infección con el virus de la enfermedad de Aujeszky (67). Estas conclusiones se basan en experimentos en los que Las CMN de cerdos inmunizados proliferan en presencia del virus y de un péptido sintético del mismo virus. El anális de las poblaciones responsables de

esta respuesta mostró que sólo los linfocitos CD4+CD8+ y no los CD4+CD8- participan. De esta menera, las CMN de los cerdos inmunizados cultivados simultáneamente con linfocitos B autólogos favorecen la producción de anticuerpos específicos contra el virus. Mientras más CMN, mayor fue el título de anticuerpos. Esto supone que dentro de la población de CMN, los linfocitos CD4+CD8+ cooperan con los linfocitos B para la producción *in vivo* de anticuerpos (67).

Sin embargo, no sólo los anticuerpos han permitido la caracterización celular, con el uso de las lectinas la caracterización de las células del sistema inmune se ha facilitado grandemente. Las lectinas son proteínas que reconocen específicamente glicanos unidos a los lípidos o a las cadenas polipeptídicas en la superficie celular. Las lectinas que reconocen específicamente glicoproteínas con estructuras O-glicosiladas se han utilizado para caracterizar poblaciones de timocitos y linfocitos. La lectina de cacahuate (*Arachis hypogaea)* reconoce y aglutina timocitos inmaduros y linfocitos T activados (68-71). Esta característica permite aislar timocitos inmaduros de la corteza tímica (68). Por otra parte, la lectina de amaranto (*Amaranthus leucocarpus)* tiene la capacidad de reconocer timocitos de la médula timica (72) y linfocitos humanos con fenotipo de célula virgen (73). La lectina de cacahuate (PNA) y de amaranto (ALL) reconocen con alta afinidad el disacárido Galß1,3GalNAc, una secuencia que se encuentra principalmente en glicanos O-glicosilados (74). El disacárido Galβ1,3GalNac se puede modificar con la actividad de enzimas específicas y la adición de ácido siálico a la secuencia Galβ1,3GalNac, que provoca inhibición de la unión de PNA a este disacárido (75). Utilizando esta lectina, en trabajos recientes, se ha sugerido que durante los procesos de activación de linfocitos T la membrana sufre una modificación en el arreglo tridimensional y en la distribución de las estructuras glicosídicas, lo que sugiere que los carbohidratos de la membrana podrían ser utilizados para realizar la caracterización fenotípica de las poblaciones en diferentes estados de maduración y/o activación.

## CAPITULO I. Respuesta inmune a rubulavirus porcino.

### Introducción

La información que hasta ahora existe con relación a la respuesta inmune de cerdos infectados con el rubulavirus porcino es escasa y únicamente existen reportes de la respuesta humoral, basados en la presencia de anticuerpos inhibidores de la hemaglutinación y neutralizantes. La enfermedad por el rubulavirus es autolimitante cuando las granjas afectadas cierran la entrada a animales de reemplazo, ya que no aparecen nuevos brotes a diferencia de producciones abiertas, donde los nuevos brotes continúan afectando principalmente las cerdas primerizas (2). En este trabajo se pretende identificar las principales características de la respuesta inmune ante la infección por el rubulavirus porcino, en virtud de que no existe hasta el momento información sobre esta enfermedad en particular.

Los virus son agentes infecciosos que se multiplican intracelularmente e inician su proceso de infección al reconocer un receptor específico en una célula blanco (76), el cual varía de acuerdo al tipo de virus involucrado; en el caso de los paramixovirus se ha demostrado que reconocen moléculas que contienen ácido siálico. El receptor del virus del sarampión es el CD46 (77), mientras que el rubulavirus porcino-LPM reconoce preferentemente moléculas que contienen sialil alfa-2,3-lactosa (15). Una vez que el virus reconoció el receptor en la célula blanco, en el caso de los virus con envoltura, se internaliza fusionándose con la membrana plasmática, y dentro de la célula el virus inicia la transcripción de sus proteínas utilizando sus propias enzimas y las de las células infectadas, que le permiten la producción de partículas víricas capaces de infectar a nuevas células blanco (78).

Existen diferentes virus capaces de infectar células del sistema inmune y de esta manera evadir la respuesta del huésped aumentando la severidad de la infección. El mejor ejemplo es el virus de la inmunodeficiencia humana. En el caso

de los Paramixovirus, se ha reportado que el virus del sarampión es inmunosupresor, capaz de infectar principalmente a los monocitos y a linfocitos, ocasionado una inmunodeficiencia transitoria (79). Una respuesta inmune celular y humoral eficaces son necesarias para el control y erradicación de las infecciones virales, la cual depende del tipo de virus, la vía de entrada, la dosis infectante, el tipo de célula presentadora y el estado general del huésped (80). En términos generales, la respuesta inmune frente a los virus se inicia con la producción de interferón-$\alpha$ por las células infectadas, lo que aumenta el potencial lítico de células NK y la expresión del MHC-I (81). El aumento en la expresión del MHC-I facilita la acción de los linfocitos T citotóxicos CD8 (82), los cuales son importantes en la erradicación del virus del sarampión (83) así como de otras infecciones virales. Se ha demostrado La presencia de linfocitos T CD4 con actividad citotóxica en diferentes modelos de infecciones virales, y se sugiere que juegan un papel importante para el control de la infección (84).

El resultado de la actividad citotóxica de las diversas efectoras (NK, linfocitos CD8 o CD4) sobre las células infectadas provoca que se liberen antígenos virales endógenos, los cuales pueden ser fagocitados, procesados y presentados como antígenos exógenos por las células presentadoras de antígeno, lo que induce la proliferación de linfocitos cooperadores CD4+ que responden con la producción de citocinas como la IL-2 e interferón-$\gamma$ o IL-4 e IL-10. Durante la infección aguda no es posible discriminar entre una respuesta Th1 o Th2, ya que predomina la producción de ambas; es en los procesos crónicos que es posible identificar un patrón de producción Th2, que favorecen muchas de las infecciones persistentes (85).

Existen diversos mecanismos efectores que participan en el control y erradicación de las infecciones virales, pero la eficacia de cada una de ellas dependen del virus involucrado y de la susceptibilidad del hospedador. Es frecuente observar que la respuesta específica contra antígenos virales provoque daño inespecífico al tejido circundante. Este evento se ha observado en el momento que se establece una respuesta citotóxica específica contra el virus del

sarampión, la cual se caracteriza por producir exantema generalizado y meningitis esclerosante (86).

El objetivo de este trabajo es identificar las principales características de la respuesta inmune del cerdo contra la infección por el rubulavirus porcino. Los resultados están presentados en el siguiente trabajo: Immunity to porcine rubulavirus infection in adult swine. Veterinary Immunology and Immunopathology 64 (1998) 367-381.

**Resultados y discusión**

Este trabajo demuestra que la infección experimental con el rubulavirus induce la presencia de anticuerpos neutralizantes a partir de la 1ª semana de infección e inhibidores de la hemaglutinación después de la 2ª semana. Los títulos más elevados se presentaron después de la 3ª y 4ª semana de infección. El análisis de la especificidad de los anticuerpos reveló que durante las primeras semanas de infección los anticuerpos están dirigidos contra la glicoproteína HN. En la 4ª y 5ª semana de infección se detectó la presencia de anticuerpos contra las proteínas M y NP, respectivamente. No obstante que otras proteínas fueron reconocidas, la glicoproteína HN se mantuvo como inmunodominante durante todo el experimento. La glicoproteína F no fue reconocida por los anticuerpos probablemente por su pobre inmunogenicidad (87). Con base en estos resultados, se propone a la proteína HN como un buen candidato para la elaboración de vacunas que ayuden al control de la enfermedad. De hecho, en nuestro laboratorio se ha purificado la proteína HN (88) y se ha realizado la síntesis de algunos epítopes inmunogénicos diseñados por algoritmos computacionales, los cuales son reconocidos por sueros de cerdos infectados (89).

Estos resultados coinciden con otros trabajos en los cuales la respuesta inmune a otros paramixovirus, como el virus de parainfluenza 3, genera anticuerpos dirigidos contra la proteína HN que se identifican desde el primer contacto con el virus y aumentan progresivamente con las reinfecciones (87). Por

otro lado, los anticuerpos contra la proteína F se detectan sólo después de múltiples infecciones. En el caso del virus de la parainfluenza se ha observado que el título de anticuerpos contra las glicoproteínas virales correlaciona con el nivel de protección (87).

Hasta la fecha, los reportes relacionados con la respuesta inmune celular al rubulavirus porcino refieren observaciones clínicas que sugieren que la enfermedad es autolimitante, ya que después de algún tiempo desaparecen los signos clínicos y no es posible detectar anticuerpos contra el virus en los animales que permanecen en la granja (3). En este trabajo se analizó la respuesta proliferativa de las células mononucleares (CMN) de cerdos infectados y testigos, a las lectinas mitogénicas fitohemaglutinina (PHA) y concanavalina A (Con A). Los resultados muestran diferencias significativas entre los cerdos infectados y testigos durante las dos primeras semanas de infección. A partir de la 3ª semana de infección la respuesta a las lectinas no presentó cambios en ninguno de los grupos. Resultados similares se han observado con el virus del sarampión, y se ha propuesto que el defecto se debe a la ausencia de citocinas coestimuladoras como la IL-2, ya que la administración *in vitro* de esta citocina restaura la proliferación de las CMN (90, 91). En la enfermedad de Aujeszky, además se ha propuesto la presencia de algunos factores solubles inducidos por el virus que inhiben la proliferación de las CMN (92). Por otro lado, se ha demostrado que la infección de los monocitos con el virus del sarampión puede ser otra causa de la baja respuesta proliferativa a lectinas mitogénicas (93, 94).

La respuesta proliferativa de las CMN al rubulavirus porcino se identificó a partir de la 2ª semana de infección. La respuesta proliferativa más alta de las CMN al rubulavirus se presentó en la 4ª semana de infección y en las siguientes semanas disminuyó gradualmente. El análisis del fenotipo de las células que proliferan en presencia del virus mostró linfocitos CD4+CD8+ y CD4+CD8- Sin embargo el incremento en los porcentajes de las células dobles positivas fue mayor al de los CD4+CD8- en comparación con el testigo. Estos resultados

coinciden con trabajos previos donde se observa incremento en los porcentajes de linfocitos estimulados con virus o parásitos (42-44).

Estudios realizados en cerdos infectados con el virus del síndrome respiratorio y reproductivo del cerdo (PRRS) (95), con el virus de la fiebre porcina africana (FPA) (96) y con el virus de la enfermedad de Aujeszky (97. 98), han revelado cambios en las poblaciones celulares de sangre periférica debidos a la infección. Los resultados de estos estudios revelan una disminución en los porcentajes de linfocitos CD4+CD8- y aumento de los linfocitos CD4-CD8+. Es importante hacer notar que ninguno de estos estudios ha evaluado la población de linfocitos CD4+CD8+. En los cerdos infectados con PRRS, los autores además de analizar los linfocitos CD4+CD8- y CD4-CD8+ determinaron la proporción CD4/CD8 de los linfocitos. Sus resultados indican que la proporción disminuyó debido al decremento de los linfocitos CD4+CD8- y al aumento de linfocitos CD4-CD8+, por lo que los autores sugieren la existencia de ciertos estímulos fisiológicos en los cerdos infectados con PRRS que inducen el incremento en los linfocitos CD4-CD8+ (95). Cerdos infectados con el virus de la FPA presentaron la misma tendencia en las poblaciones celulares (96) con una disminución en los linfocitos CD4+CD8- y el aumento de los CD4-CD8+ en la infección aguda y el aumento de ambas poblaciones en la infección crónica. Los autores sugieren que en la infección aguda la actividad citotóxica de los linfocitos CD4-CD8+ no requiere la participación de los linfocitos cooperadores, como sucede con la infección por citomegalovirus en roedores en donde la eliminación de células CD4+CD8- no afecta el control del virus (99). Por el contrario, en la infección crónica es necesaria la participación de ambas poblaciones celulares.

Los cambios en las poblaciones celulares durante la infección con el rubulavirus fueron similares a otras infecciones virales del cerdo, en las cuales se presenta un aumento en la población de linfocitos CD4-CD8+ y una disminución en la población CD4-CD8+. Nuestros resultados indican que los linfocitos CD4-CD8+ se incrementaron en la 1ª semana de infección, mientras que los porcentajes de linfocitos CD4+CD8- disminuyen gradualmente durante la

infección. Una posible explicación a la disminución de los linfocitos CD4+CD8- sería que el virus estuviera eliminando esta población celular, como sucede con el virus del SIDA (100). Sin embargo hemos analizado la susceptibilidad de las células no adherentes y el virus se une al 19% de esta población sin multiplicarse en ella, por lo que se puede suponer que este fenómeno no se presenta en la infección por el rubulavirus porcino. Otra posibilidad es el reclutamiento celular en los órganos blanco de la infección, como se ha propuesto en otras infecciones virales (101). En cuanto el incremento gradual de la población CD4+CD8+ durante la infección, en primer lugar, podría deberse a comportamiento normal de esta población, ya que previamente se ha reportado que esta población incrementa con la edad del animal (42). Sin embargo, ya que los cerdos infectados presentaron mayor aumento que los no infectados, esta posibilidad es poco probable. Otra posibilidad es que el incremento refleje mayor memoria inmune frente al rubulavirus y en consecuencia mejor control de la enfermedad.

En este trabajo se presentan los primeros reportes de la respuesta celular y humoral al rubulavirus porcino. Los resultados demuestran que la proteína HN del rubulavirus porcino es el antígeno inmunodominante del virus. Además, se presentan resultados que suponen la importancia de los linfocitos CD4+CD8+ en la respuesta inmune al virus. Estudios posteriores serán necesarios para eterminar la importancia de estas células en el control de la infección.

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# Immunity to porcine rubulavirus infection in adult swine

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**Abstract**

The immune response against the porcine rubulavirus was analyzed in experimentally infected adult pigs. High titers of virus neutralizing and hemagglutinating inhibitory antibodies were identified in infected animals. The antibody specificity was directed towards HN, M, and NP rubula virion proteins; immunodominance of HN proteins was demonstrated. Peripheral blood mononuclear cells from infected, but not from non-infected pigs proliferated in vitro in response to virus antigenic stimuli, showing a bell-shaped plot with the highest peak at 5 weeks post-infection. Virus-induced lymphoblasts expressed $CD4^+CD8^+$ phenotype, whereas lectin-induced lymphoblasts were mainly identified as $CD4^+CD8^-$ cells. Phenotype analysis of freshly prepared PBMC revealed increased number of both monocytes ($PoM1^+$) and total T lymphocytes ($CD2^+$) early during infection, with reduced values of B lymphocytes at 4 weeks post-infection. Decrease in $CD4^+CD8^-$ blood cells was observed at 3 weeks post-infection, whereas both $CD4^-CD8^+$ and $CD4^+CD8^+$ cells increased 1 and 4 weeks post-infection, respectively. This work discusses the relevance of $CD4^+CD8^+$ T cells in the control of porcine rubulavirus infection. 

*Keywords*: Immunity; Lymphocytes; Antibodies; Rubulavirus; Paramyxovirus; Porcine

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## 1. Introduction

Blue eye disease of swine emerged in La Piedad Michoacan, Mexico (LPM) in 1980; it was characterized by encephalitis, pneumonia and corneal opacity in suckling pigs and reproductive disorders in older pigs. Clinical signs were reproduced in piglets inoculated with a virus with hemagglutinating activity isolated in 1981 (Stephano et al., 1988). Characterization of LPM virus (LPMV) isolated from a meningo-encephalitic piglet in 1984 confirmed paramyxoviral etiology of the disease. LPMV is an enveloped RNA virus, which possesses hemagglutinating, hemolytic and syncytia-forming activities (Moreno-López et al., 1986). LPMV is constituted by six structural proteins, hemagglutinin–neuraminidase (HN), fusion (F) and matrix (M) proteins are expressed at the envelope; whereas nucleoprotein (NP), phosphoprotein (P) and large (L) proteins form the nucleocapsid (Sundqvist et al., 1990). Cloning and sequencing of M (Berg et al., 1991), HN (Sundqvist et al., 1992); P (Berg et al., 1992), F (Berg et al., 1997), and L (Svenda et al., 1997) protein genes showed that LPMV is closely related to Mumps virus and Simian virus 5, supporting the classification of porcine LPMV in the *Rubulavirus* genus of *Paramyxoviridae* family (Rima et al., 1995).

A severe neuropathological syndrome developed in 3-day-old pigs inoculated with the rubulavirus, which died or were moribund from 8 to 11 days after infection. The virus was widespread in the central nervous system (CNS) (Allan et al., 1996), but the highest titers of virus were found in the midbrain of these pigs (McNeilly et al., 1997). Inoculation of porcine rubulavirus in 17-day-old pigs induced mild respiratory and nervous signs, with restricted distribution of virus antigen to olfactory bulb and midbrain structures (Allan et al., 1996).

Naturally infected adult pigs showed decreased infertility rates in gilts, stillbirths and mummified fetuses in pregnant sows and epididymitis and orchitis in boars (Stephano, 1994). A novel strain of porcine rubulavirus (Jalisco/1992) was isolated during an outbreak in a breeding farm (Ramírez-Mendoza et al., 1997). Genital tract alterations were reproduced in 9-month-old sexually mature boars inoculated with this virus, including swelling of epididymis, temporal orchitis, reduced spermatozoa concentration and motility, as well as testis atrophy. Rubulaviral antigen was recognized at the epithelium of epididymis head at 15, 30, 45, and 70 days after infection. No infectious virus nor viral antigen was identified in the CNS of these pigs (Ramírez-Mendoza et al., 1997).

Host susceptibility to viruses depends on both expression of virus receptors and virus survival to immune surveillance. We have identified that porcine rubulavirus recognizes Neuraminic acid α2,3 Galactose (NeuAcα2,3Gal) oligosaccharides, which inhibited the virus hemagglutinating activity (Reyes-Leyva et al., 1993). We found that cell culture expression of NeuAcα2,3Gal, but not its isomer NeuAcα2,6Gal, was a requirement for the porcine rubulavirus infection process (Reyes-Leyva et al., 1997). Furthermore, we have also identified a broad expression of NeuAcα2,3Gal-glycoconjugates at CNS and respiratory tissues of newborn pigs, whereas high expression of NeuAcα2,6Gal was observed in the olfactory mucosa and bulb of adult pigs, indicating that expression of specific oligosaccharide sequences seems to be correlated with tissue susceptibility to porcine rubulavirus.

Several reports suggest that porcine rubulavirus is cleared by host defenses in infected older pigs (Stephano et al., 1988; Allan et al., 1996); however, until now, no information is available on the role of immune responses in the control of procine rubulavirus infection. This work analyzes the immune response to porcine rubulavirus in infected adult pigs.

## 2. Materials and methods

### 2.1. Virus

The porcine rubulavirus strain Jalisco/1992, was obtained from the Veterinary Faculty, National University of Mexico, Mexico (Ramírez-Mendoza et al., 1997). Rubulavirus was propagated in the pig kidney cell line PK-15 with MEM (supplemented with 10% fetal bovine serum, 100 U $ml^{-1}$ penicillin and 100 µg $ml^{-1}$ streptomycin) until the cultures showed maximum cytopathic effect. Then, infected cell cultures were frozen/thawed thrice and supernatants were clarified by centrifugation at 3200 rpm for 45 min at 4°C. The virus infectivity was titrated in cell cultures in 96-well microculture plates (Falcon Labware, NJ), using serial 10-fold dilutions of virus made in MEM, and the titer was expressed in $TCID_{50}$ $ml^{-1}$ (Burleson et al., 1992). For viral antigen production, the virus in the supernatants was concentrated by centrifugation at 100 000 *g* for 4 h at 4°C, filtered through 0.45 µm membranes, aliquoted and stored at −70°C until use. Negative antigen control was similarly prepared with non-infected cell culture supernatants (Ramírez-Mendoza et al., 1997). Protein determination was made by the method of Bradford (1976).

### 2.2. Antibodies

Mouse monoclonal antibodies (MAb) specific for porcine cluster of differentiation (CD) molecules were purchased from VMRD (Washington, DC), these were: MAb MSA4, IgG2a anti-CD2; MAb 74.12.4, IgG2b anti-CD4; MAb 76.2.11, IgG2a anti-CD8; MAb PG130A, IgM anti Po-M1; and MAb PIg45A, IgG2b anti-IgM. Fluorescein isothyocyanate (FITC)-conjugated goat polyclonal antibodies anti-mouse IgG; phycoerythrine (PE)-conjugated rat MAb anti-mouse IgG2a, FITC-conjugated rat MAb anti-mouse IgG2b, and FITC-conjugated goat polyclonal antibodies anti-mouse IgM were purchased from Becton & Dickinson (Mountain View, CA).

### 2.3. Experimental design

Ten 9-month-old male, York Landrace hybrid pigs were used. They were born at a 'blue eye' disease-free farm and were free of porcine rubulavirus and pseudorabies virus as assessed by the lack of specific serum antibodies in both virus neutralization and indirect immunofluorescence tests. Seven boars were intranasally inoculated with 5 ml of rubulavirus ($10^4$ $TCID_{50}$ $ml^{-1}$) and placed individually in an isolation facility. Blood

samples for both serum and cell preparations were taken 1 week before and each week after inoculation, for 7 weeks post-infection (p.i.).

### 2.4. Serological test

Hemagglutination inhibition (HI) and virus neutralization (VN) tests were carried out with sera from infected and non-infected pigs, according to standard procedures used for diagnosis of rubulavirus porcine diseases (Ramírez-Mendoza et al., 1996). Briefly, for the HI test, sera were heat-inactivated at 56°C for 30 min and treated with 25% acid-washed kaolin to remove non-specific inhibitors, then, sera (50 μl) were titrated by serial 2-fold dilutions in phosphate-buffer saline (PBS, pH 7.2). HI test was performed using 0.5% bovine erythrocytes (50 μl) and virus antigen (50 μl) with eight hemagglutinating units (HAU, titre=8) in U-well plates (Falcon). The HI titre was considered with the last dilution of serum that completely inhibited the eight HAU of the virus. For VN assays, sera were diluted in MEM supplemented with 4% fetal bovine sera. Serial 2-fold dilutions of sera were performed in 96- well flat bottom microtitre plates and mixed with an equal volume (50 μl) of rubulavirus containing 300 $TCID_{50}$. The plates were then incubated for 60 min at 37°C. Finally, 200 μl of PK-15 cell suspension ($10^4$ cells/well) was added to each well and incubated for 72 h at 37°C. The end point was determined by HAU in the infected cells' supernatants. Equal volumes (50 μl) of supernatant fluid and 0.5% bovine erythrocytes were mixed in a U-well microtitre plate. The VN titre is considered with the last dilution of serum that completely inhibited hemagglutinin production of the virus.

### 2.5. Western-blot assays

Virus proteins (100 μg $ml^{-1}$) were separated by SDS-PAGE (Laemmli, 1970) and transferred to 0.22 μm nitrocellulose membranes (Towbin et al., 1979). Membranes were treated for 1 h at 37°C with washing buffer (WB) (0.1 M phosphate, 0.5 M sodium chloride, 5% fat-free milk, 0.5% Tween 20, pH 7.2). For immunodetection, membranes were incubated with a 1:25 dilution of the test sera, 1 h at room temperature, washed with WB, incubated with biotin-labeled rabbit anti-porcine IgG diluted 1:150, 1 h at room temperature, washed with WB, incubated with 1:3000 dilution of streptavidin–horseradish peroxidase conjugate, 30 min at room temperature, washed with WB, and finally incubated with NBT–BCIP substrate.

### 2.6. Lymphocyte proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll–Hypaque 1077 (Sigma, St. Louis, MO) gradient centrifugation (density=1.067) and cultured at $1.5\times10^5$ $well^{-1}$ in 96-well flat bottom plates in HEPES-buffered RPMI1640 culture medium, supplemented with $5\times10^{-5}$ M 2-mercaptoethanol, 2 mM sodium pyruvate, 2 mM L-glutamine, 100 U $ml^{-1}$ penicillin, 100 μg $ml^{-1}$ streptomycin, 1 μg $ml^{-1}$ gentamycin, 10% non-essential amino acids and 10% fetal bovine serum (Kimman et al., 1993). PBMC were stimulated with 10 μg $ml^{-1}$ of viral antigen previously inactivated by

heating for 10 min at 90°C. Lack of infectious virus was subsequently checked by cell culture. The cells were stimulated for 5 days at 37°C, in a humidified incubator with 5% $CO_2$. Negative antigen was used for mock stimulation. Phytohemagglutinin (PHA, 8 μg $ml^{-1}$) and Concanavalin A (Con A, 1 μg $ml^{-1}$) (Sigma) were used as positive controls; 1 μCi of $^3$H-thymidine (Specific activity=6.7 Ci/mmol; New England Nuclear, Boston, MA) was added during the last 18 h of culture (Kimman et al., 1993). Cells were harvested onto glass fiber filters and radioactivity incorporated into DNA was measured in a Beckman LS6000SE SE-counter (Beckman, Fullerton, CA). Lymphoblasts obtained after stimulation of PBMC with virus or lectins were processed for double-stained flow cytometry assays, to identify expression of CD4 and CD8 molecules on these cells.

### 2.7. *Single and double cytofluorometric (CF) analysis*

Phenotype of freshly prepared PBMC or lymphoblasts was determined as in Summerfield et al. (1996). Briefly, for single CF analysis, cells were incubated with mouse MAb specific towards CD2 (MAb MSA4), Po-M1 (MAb PG130A) or IgM (Plg45A); followed by incubation with FITC-conjugated goat polyclonal antibodies against mouse IgG. For double CF analysis, cells were incubated with MAbs anti-CD8 (76-2-11) and anti-CD4 (74.12.4), followed by incubation with both FITC- and PE-conjugated isotype-specific rat MAbs. All incubations were carried out at 4°C for 15 min. Cells were washed with 0.1 M PBS, pH 7.2, 0.2% BSA, 0.1% $NaN_3$. Stained cells were analyzed by cytofluorometry, (FAS Calibur Becton & Dickinson, Mountain, View, CA) as described.

## 3. Results

### 3.1. *Clinical signs*

Infection of sexually mature pigs with the porcine rubulavirus induced the clinical signs of reproductive disease, which consisted in epididymis and testis swelling. Neither neuropathological nor other lesions were observed in these animals, as previously described (Ramírez-Mendoza et al., 1997). The presence of circulating virus was determined by immunofluorescence assays on blood slides, and viremia was identified from 6 to 15 days p.i. (data not shown).

### 3.2. *Antibody response*

Induction of antibody response during rubulavirus infection in adult swine was determined by VN and HI tests. Seroconversion started at 1 week p.i. with the production of VN antibodies, which ranged from 4 to 6 $log_2$ titers during the first 4 weeks p.i. VN titers increased up to 8.5 $log_2$ at 5 weeks p.i., remaining at high titers until the end of the study. HI antibodies were identified at the second week p.i., with 5 $log_2$ titers. In contrast to the VN response, HI titers remained in a plateau throughout the study (Fig. 1).

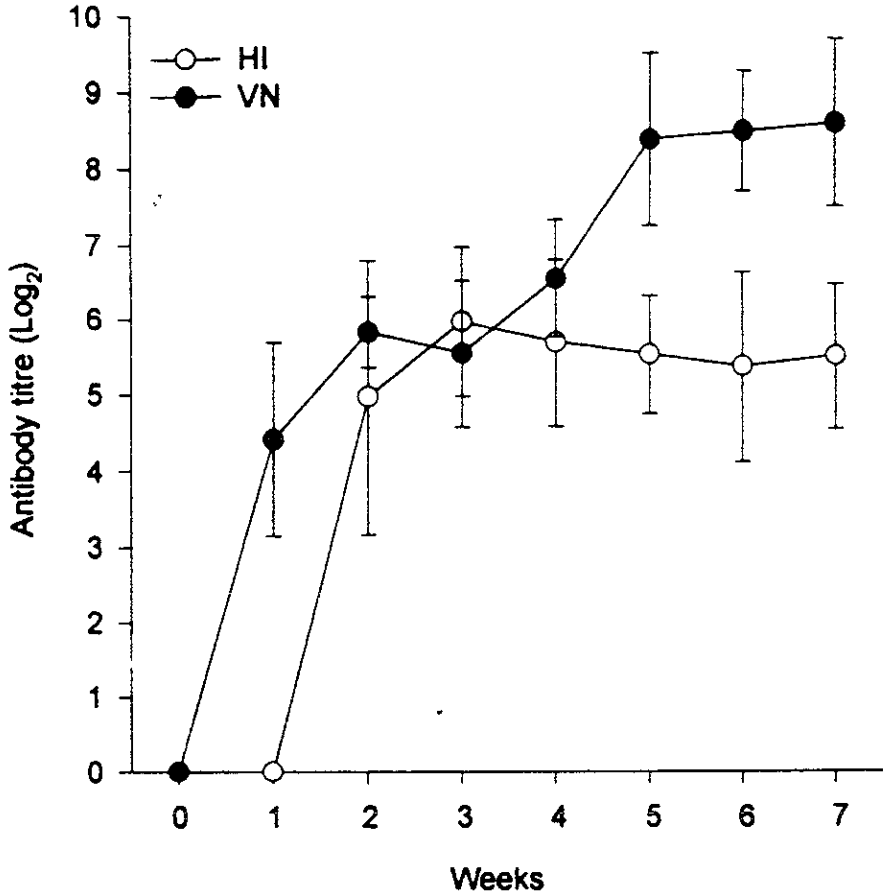


Fig. 1. Antibody response from rubulavirus infected pigs. Sera from infected pigs ($n$=7) were analyzed by VN and HI assays each week after infection.

### 3.3. Antibody specificity

To characterize the specificity of antibodies induced by the virus and the time at which each viral protein antigen was recognized, rubulavirus proteins were separated by SDS-PAGE and Western blot assays were performed with sera from infected pigs, obtained weekly. These assays revealed antibody response against 68, 66, and 40 kDa antigens, corresponding to NP, HN, and M proteins. Western blot assays revealed antigenic predominance of HN glycoprotein in the antibody response triggered against rubulavirus infection, since HN glycoprotein was recognized by all infected pigs. Six of the seven infected pigs recognized HN glycoprotein at 2 weeks p.i. at 3 weeks p.i. all pigs had responded and remained seropositive hereafter (Fig. 2). Response against M protein was identified from 4 weeks p.i. by three out of the seven infected pigs. Antibodies against NP protein were identified in four pigs at 5 weeks p.i., and in six pigs at 6 and 7 weeks p.i.; one pig remained seronegative to NP. Antibodies against F, P, or L proteins were not identified in the Western blot under the conditions of these assays.

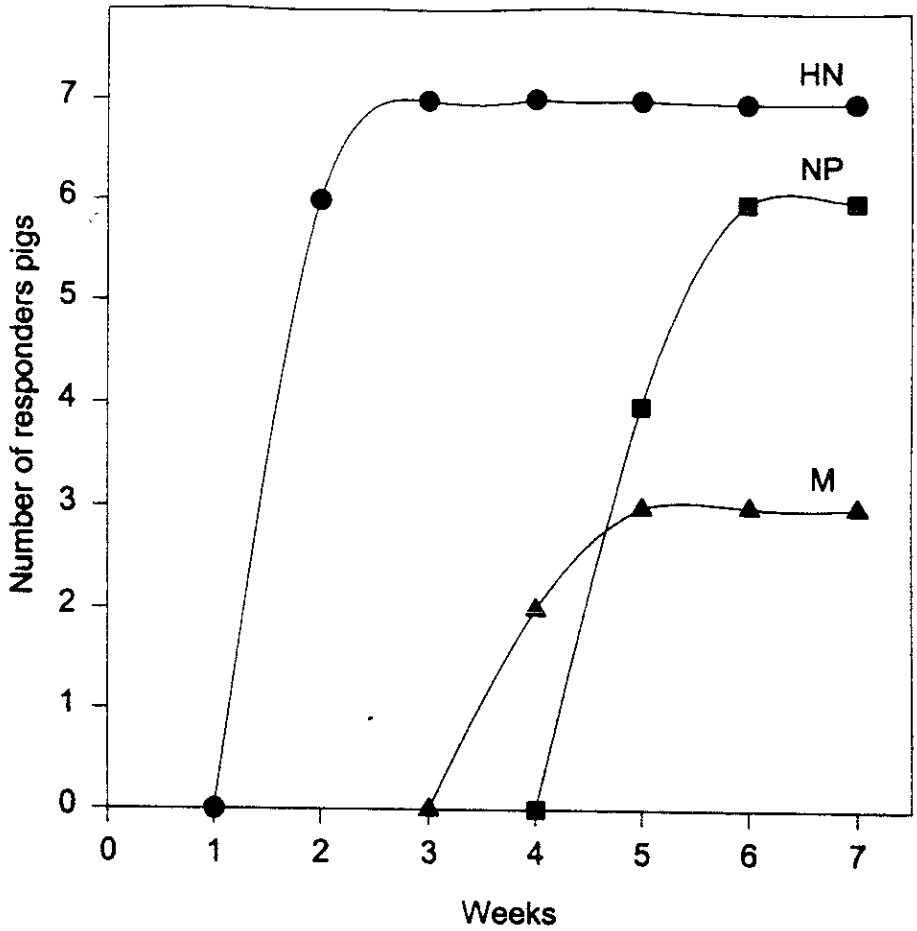


Fig. 2. Kinetics of immune response measured by the number of pigs showing specific antibody response to HN, NP, and M after infection with porcine rubulavirus. Porcine rubulavirus proteins were separated by SDS-PAGE and analyzed by Western blot assays with sera from infected pigs obtained weekly.

### 3.4. *Lymphocyte proliferation induced by lectins*

The status of the cellular immune response was analyzed in lymphoproliferative assays using lectins. We prepared PBMC from control and rubulavirus-infected pigs to evaluate their capacity to proliferate in response to T mitogenic lectins. Cells were cultured in the presence of PHA or Con A lections during 72 h and proliferation was determined by incorporation of $^{3}$H-thymidine. Results are expressed as stimulation indexes (SI=cpm of stimulated cells/cpm of unstimulated cells). The plots of proliferative responses obtained after stimulation of PBMC with PHA or Con A (Fig. 3) were similar among infected and control pigs. PHA- or Con A-stimulated PBMC obtained from non-infected pigs showed proliferation values ranging from 40 to 80 SI throughout the study. The proliferative responses induced by both PHA and Con A in PBMC from infected pigs were irregular along time; however, three events were consistent: (a) A lack of proliferative response at 1 week p.i. (SI<20), which represented a significant difference of $p<0.005$ with respect to uninfected pigs; (b) SI (>80) higher than those of non-infected pigs were measured at 2–5 and 7 weeks p.i.; and (c) a decline in the proliferative response at 6 weeks p.i. (Fig. 3).

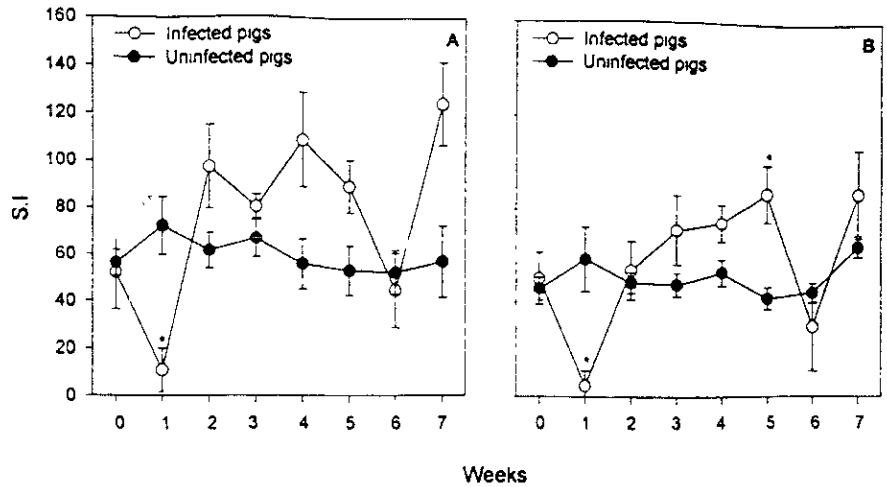


Fig. 3. Mitogen induced lymphoproliferative responses. PBMC from non-infected pigs (filled circles, $n$=3) and infected pigs (open circles, $n$=7), were stimulated with Con A (A) and PHA (B) lections. Response was evaluated by SI 18 h after $^3$H-thymidine incorporation. SI=cpm$\times 10^3$ stimulated cells/cpm$\times 10^3$ unstimulated cells. The cpm in PBMC from unstimulated pigs was <900. $^*p<0.005$.

### 3.5. *Lymphocyte proliferation induced by viral antigen*

The optimal conditions for virus-induced lymphoproliferative response were established previously, the highest SI was obtained by incubation of PBMC in the presence of 10 $\mu$g ml$^{-1}$ of virus antigen for 5 days (data not shown); these conditions were applied in this study. In order to identify the induction of cellular immune response through infection, PBMCs were prepared from infected and non-infected pigs and cultured in the presence of both viral or negative antigen. The immunogenicity of porcine rubulavirus antigen was confirmed by the induction of proliferative response in PBMC from infected pigs stimulated with viral-recall antigen; this response was apparent at 2 weeks p.i., showing a peak at 4 weeks p.i., when proliferation had reached a mean of 14±2 SI (Fig. 4). PBMC from non-infected pigs did not proliferate after incubation in vitro with rubulavirus antigen.

### 3.6. *Virus-induced lymphoblasts expressed CD4$^+$CD8$^+$ phenotype*

Phenotype analysis was performed to identify predominance of single positive (SP) CD4$^+$, (SP) CD8$^+$, or double positive (DP) CD4$^+$CD8$^+$ T cell subpopulations proliferating in response to rubulaviral antigen. Analysis of cell phenotype was determined by double-strained flow cytometry after 5 days of viral-antigen stimuli. PHA- and mock-stimulated PBMC were used as positive and negative controls, respectively. Resting (small lymphocytes) (Fig. 5, R1) and activated cells (lymphoblasts) (Fig. 5, R2) in the same culture were independently analyzed for their phenotype based on their characteristic light scatter profile (Fig. 5, top). After this, cell phenotypes were determined by contour plots. Small lymphocytes (R1) did not reveal differences between virus or PHA stimulated PBMC (Fig. 5, middle: B and C, respectively). In virus-induced

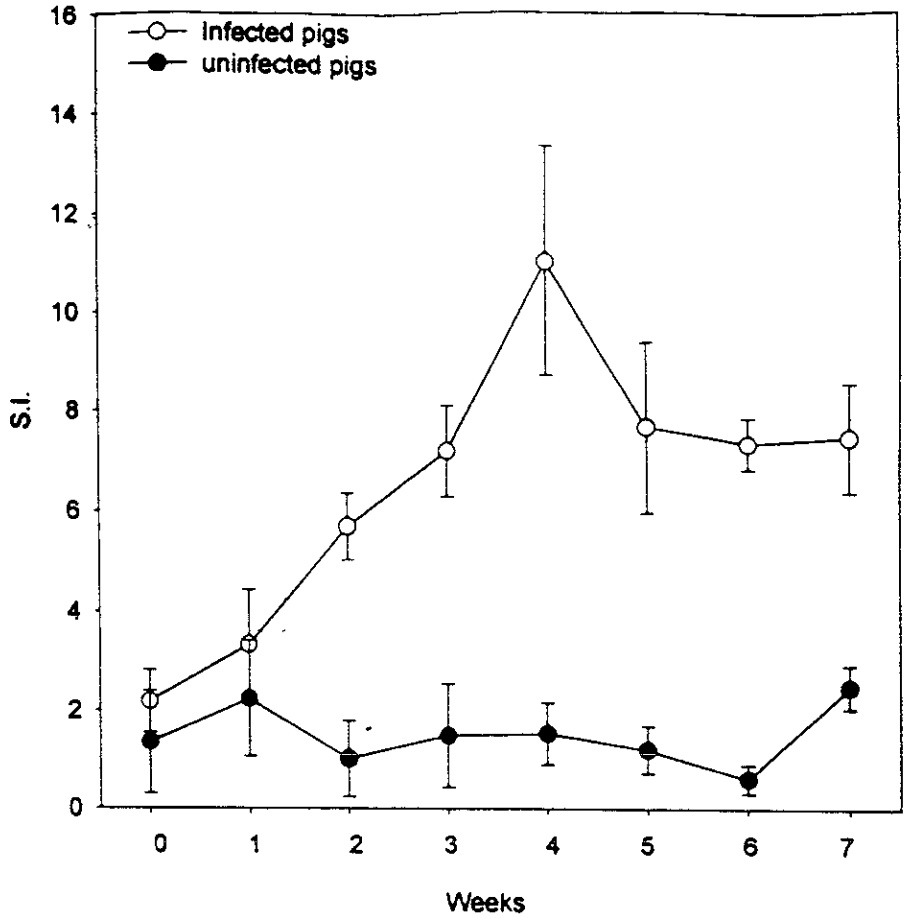


Fig. 4. Virus-induced lymphoproliferative responses. PBMC from infected (open circles, $n$=7) and non-infected pigs (filled circles, $n$=3) were cultured in the presence of virus antigen. SI was evaluated 18 h after $^3$H-thymidine incorporation. SI=cpm$\times 10^3$ stimulated cells/cpm$\times 10^3$ unstimulated cells. The cpm in infected pigs in the presence of mock virus, as well as in uninfected pigs in the presence of viral antigen was <1200. $^*p<0.005$.

lymphoblasts (R2) we identified 39% of SP $CD4^+$, 14% of SP $CD8^+$, and 21% of DP $CD4^+CD8^+$ cells (Fig. 5, bottom: B), the two first values corresponded to an increase of 95% and 110%, while the latter, represents a reduction with respect to mock-induced lymphoblasts (Fig. 5, bottom: A). In contrast, phenotyping of PHA-stimulated PBMC revealed 57% of SP $CD4^+$, 22% of SP $CD8^+$, and 9% of $CD4^+CD8^+$ cells (Fig. 5, bottom: C). The values of $CD4^+$ cells correspond to an increment of 185% with respect to mock-stimulated lymphoblasts. In addition, it is noteworthy that DP cells did not proliferate in response to PHA stimuli, as occurred in virus.

### *3.7. Identification to T, B, and macrophage cells in PBMC from infected pigs*

The relative values of blood mononuclear cell populations, i.e. $CD2^+$ T cells, $IgM^+$, B cells and $PoM1^+$ monocytes/macrophages were determined 1 week before and 4 weeks after infection. For cell identification, single color cytofluorometric analysis was performed in freshly prepared PBMC. Both infected and non-infected pigs showed

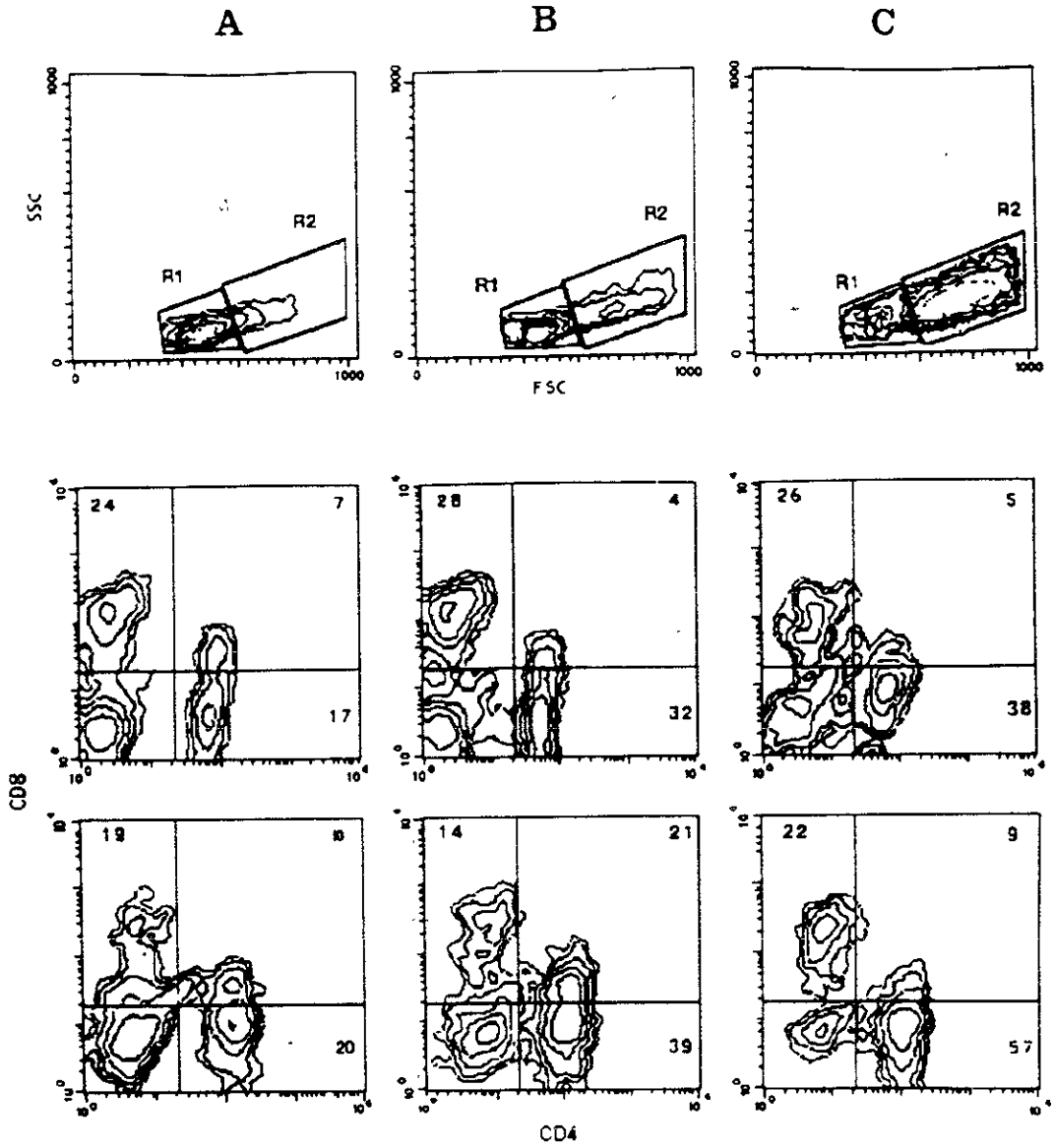


Fig. 5. Phenotyping of virus-induced lymphoblasts in PBMC from rubulavirus-infected pigs. PBMC were cultured with virus-antigen (B), PHA (C) or with mock stimuli (A); incubated with anti-CD4 (74.1.24) and anti-CD8 (76.2.11) MAb and their size analyzed (forward scatter, FCS) vs. granular contents (side scatter, SSC) (top). Resting cells were gated in region 1 (R1, middle) whereas lymphoblasts in region 2 (R2, bottom). CD4 and CD8 percentages were determined by contour plot.

similar values of $CD2^+$, $IgM^+$, and $PoM1^+$ cells in the blood samples obtained before infection. Virus infection induced an in vivo T cell proliferative response that was evidenced by the increase of $CD2^+$ cells in infected pigs, when compared with control ones (Table 1). The values of B lymphocytes $IgM^+$ in infected pigs were similar to those of non-infected pig during the first two 2 weeks p.i. however, a significant decrease in circulating B cells was observed at 3 and 4 weeks p.i. (Table 1). The values of $PoM1^+$ cell population were the most variable after rubulavirus infection. $PoM1^+$ cells increased

Table 1
The relative values of blood mononuclear cell populations were determined by flow cytometry

| Weeks | T Lymphocytes | | B Lymphocytes | | Monocytes | |
|---|---|---|---|---|---|---|
| | U | I | U | I | U | I |
| 0 | 39.0±2.0 | 39.0±2.0 | 27.0±2.1 | 27.6±5.2 | 16.0±2.6 | 17.6±3.3 |
| 1 | 41.5±1.8 | 50.2±6.1 | 27.0±2.6 | 25.0±4.4 | 17.3±4.6* | 32.6±5.6 |
| 2 | 40.3±2.5 | 44.7±4.3 | 25.6±4.0 | 29.5±3.2 | 16.5±4.9 | 8.24±4.4 |
| 3 | 39.3±3.0 | 32.7±3.3 | 27.0±2.0 | 19.1±3.6 | 16.3±2.3 | 7.7±4.6 |
| 4 | 41.0±3.0 | 48.7±2.7 | 29.0±2.7 | 16.0±5.0* | 14.6±2.3 | 12.3±4.2 |

PBMC from infected ($n$=7, I) and uninfected pigs ($n$=3, U), were obtained weekly to determine percentages of T lymphocytes ($CD2^+$), B lymphocytes ($IgM^+$), and monocytes/macrophages ($PoM1^+$). Flow cytometry was performed using mouse monoclonal antibodies anti-CD2; anti-IgM, and anti-PoM1 recognized by isotype-specific rat anti-mouse antibodies (For more details, see Section 2).
*=$p$<0.005.

up to 32.6% (±5.6) at 1 week p.i., but decreased to 8.24% (±4.4) and 7.7% (±4.6)% at 2 and 3 weeks p.i., respectively; at 4 weeks p.i., monocytes returned to normal values (Table 1).

### 3.8. $CD4^+CD8^+$ T cell expression in infected pigs

For T cell-subtype identification, double-stained cytofluorometric analyses were performed in freshly prepared PBMC. Infected and control pigs showed similar values of SP $CD4^+$, SP $CD8^+$, and DP $CD4^+CD7^+$ cells in the blood samples taken before infection (Fig. 6). The $CD4^+CD8^-$ cells did not change at the first 2 weeks p.i. in infected and non-infected pigs; however, at 3 weeks p.i. the values in infected pigs

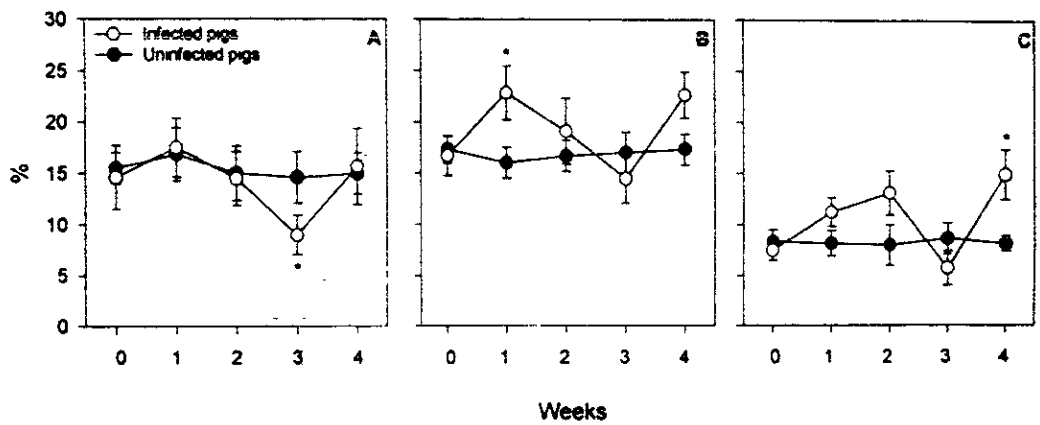


Fig. 6. The relative values of T lymphocyte subsets from blood mononuclear cells determined by flow cytometry. PBMC from infected (open circles, $n$=7l) and non-infected pigs (filled circles, $n$=3), were obtained weekly to determine percentages of $CD4^+CD8^-$ (A), $CD4^-CD8^+$ (B), and $CD4^+CD8^+$ (C), using mouse monoclonal antibodies anti-CD4, and anti-CD8 recognized by isotype-specific rat anti-mouse antibodies.
*$p$<0.005.

decreased to 8.98±1.92 ($p<0.005$), which corresponds to $0.9\times10^6$ $CD4^+CD8^-$ cells $ml^{-1}$ of blood. At the end of the experiment, this cellular population increased and showed similar numbers to those found in control-animals ($1.6\times10^6$ cells $ml^{-1}$ of blood). An increase in $CD4^-CD8^+$ cells was observed at the first week p.i., thereafter this population decreased presenting the lowest levels at the third week. The DP $CD4^+CD8^+$ cells from infected pigs increased at first and second week p.i. (from $0.8\times10^6$ to $1.3\times10^6$ cells $ml^{-1}$), although this increment does not represent a significant difference ($p>0.005$). At the third week p.i., this population of cells presented the lowest percentages in infected pigs; however, at the fourth week p.i., the percentages of $CD4^+CD8^+$ in infected pigs were 14.91±2.45 ($1.5\times10^6$ cells $ml^{-1}$), whereas, in uninfected pigs, they were 8.16±0.76 ($p<0.005$) ($0.8\times10^6$ cells $ml^{-1}$).

## 4. Discussion

The structural and biological characteristics of porcine rubulavirus have been described previously (Stephano et al., 1988; Linné et al., 1992). This virus produces acute meningoencephalitis in suckling pigs (Stephano et al., 1988; Allan et al., 1996), whereas a non-fatal reproductive syndrome is shown in infected adult pigs (Ramírez-Mendoza et al., 1997). Our group has been interested in establishing the mechanisms involved in the susceptibility and resistance to porcine rubulavirus; indeed we have identified the specificity of porcine rubulavirus towards oligosaccharide structures and their role in the infection process (Reyes-Leyva et al., 1993, 1997). In this work, we describe, for the first time, some parameters concerning antibody and T cell response during experimental infection of adult pigs. The antibody response was monitored with VN and HI tests. Serum-neutralizing antibodies were detected as early as 1 week p.i., but the highest titers were observed after 4 weeks p.i. Antibodies able to inhibit the hemagglutinating activity of the virus were detected at 2 weeks p.i., presenting low and constant titers during the experiment. Presence of specific antibodies towards rubulavirus proteins, with high titers until 1 year after the occurrence of natural infection, have been described, suggesting the relevance of antibodies in the control of infection (Stephano et al., 1988). In infections induced by other paramyxoviruses, such as parainfluenza virus type 3, the presence of serum-neutralizing antibodies has also been correlated with the resistance to infection (Chanock and McIntosh, 1990). The specificity of antibodies was further characterized by Western blot analysis, which showed that all infected pigs recognized the HN glycoprotein from 2 weeks p.i. and thereafter. Some infected pigs also recognized M and NP proteins, but at 4–6 weeks p.i., indicating that the porcine rubulaviurs HN protein is the most immunogenic protein, and may provide the antigenic basis to improve diagnosis of this rubulavirus and development of vaccination programs. Work is now in progress to characterize the HN protein and its antigenic properties (Zenteno et al., 1998).

The cellular immune response was evaluated by measuring the proliferative response of PBMC to heat-inactivated virus and to lectins and subsequently determining the phenotype of proliferating lymphoblasts. An immunosuppression phase was identified in infected animals represented by low SI of lymphocytes stimulated with Con A and PHA

during the first week p.i. Stephano et al. (1988) described a high susceptibility of rubulavirus-infected animals to secondary infections, even to other paramyxoviruses (Griffin et al., 1994), which could result from the infection produced by the rubulavirus. The specific response to the rubulavirus was observed 5 days after stimulation of PBMC with inactivated virus. This response was considered positive when SI was greater than 4. The viral-recall antigen response was observed in the infected pigs from 2 weeks p.i. and thereafter. These results indicate that rubulavirus infection induces memory lymphocytes that can be reactivated in the in vitro assays. PBMC from uninfected pigs did not proliferate in response to viral antigen, confirming the specificity of the memory response.

Phenotype analysis of freshly prepared PBMC revealed increased values of monocytes ($PoM1^+$) and total T lymphocytes ($CD2^+$) during early infection, with reduced values of B lymphocytes at 4 weeks p.i. At the end of the study, we observed a reduced number of B cells (surface $IgM^+$), probably as a result of the maturation of these cells to become antibody-producing cells, expressing surface IgG and producing high amounts of antibodies (Zinkernagel et al., 1996). The highest numbers of $CD4^-CD8^+$-positive cells were found at 1 week p.i. These T cells have been related usually to cytotoxic activity (Scott and Kaufmann, 1991), and are frequently observed at elevated percentages in several viral infections (Tripp et al., 1995). Although in this work the cytotoxic activity of $CD4^-CD8^+$ cells was not evaluated, their relevance in early response to the porcine rubulavirus cannot be excluded.

Stimulation of PBMC from infected pigs with viral antigen-induced increase $CD4^+CD8^-$ and $CD4^+CD8^+$ T lymphoblasts; in contrast, stimulation with lectins increased only $CD4^+CD8^-$, but did not affect $CD4^+CD8^+$ cells, indicating that expression of the $CD4^+CD8^+$ phenotype was driven by viral antigen stimulation. Interestingly, the numbers of $CD4^+CD8^+$ T lymphocytes also increased in the whole blood of pigs infected with porcine rubulavirus. Although, the concentration of double-positive cells showed increased numbers during almost the whole experiment, they diminished at 3 weeks p.i., along with other T cell subpopulations. This phenomenon could be attributed to arrest of effector cells in immunocompromised tissues. Although the specific rule of porcine $CD4^+CD8^+$ lymphocytes has not been clearly stated, some authors suggest that this group of cells has memory functions, since these cells are able to react in an antigen-specific secondary immune response, suggesting that memory/effector cells are present within this T lymphocyte population (Summerfield et al., 1996; Zuckermann and Husmann, 1996). We suggest that the possible effector function of $CD4^+CD8^+$ cells participates in the regulation of the immune response to rubulavirus in pigs. At the present, the possible effector function of $CD4^+CD8^+$ in rubulavirus infections is under investigation in our laboratory.

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## References

Allan, G.M., McNeilly, F., Walker, I., Linné, T., Moreno-López, J., Hernandez, P., Kennedy, S., Carroll, B.P., Herron, H., Foster, J.C., Adair, B., 1996. A sequential study of experimental paramixovirus (LPMV) infection of pigs: Immunostaining of cryostat sections and virus isolation. J. Vet. Diagn. Invest. 8, 405–413.

Berg, M., Sundqvist, A., Moreno-López, J., Linné, T., 1991. Identification of the porcine paramyxovirus LPMV matrix protein gene: Comparative sequence with other paramyxovirus. J. Gen. Virol. 72, 1045–1050.

Berg, M., Hjertner, B., Moreno-López, J., Linné, T., 1992. The P gene of the porcine paramyxovirus encodes three possible polypeptides P, V and C: The P protein mRNA is edited. J. Gen. Virol. 73, 1195–1200.

Berg, M., Bergvall, A-C., Svenda, M., Sundqvist, A., Moreno-López, J., Linné, T., 1997. Analysis of the fusion protein gene of the porcine rubulavirus LPMV: Analysis of the paramyxovirus F protein. Virus Genes 14, 57–61.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72, 248–259.

Burleson, F., Chambers, M.T., Weidbrauk, D., 1992. Virology, A Laboratory Manual. Harcourt Brace Jovanich, Academic Press, pp. 58–61.

Chanock, R.M., McIntosh, K. 1990. Parainfluenza virus. In: Fields, B.N., Knipe, D.M. (Eds.), Fields Virology, vol. I. Lippincot-Raven, Philadelphia, pp. 963–988.

Giffin, D., Ward, B., Esolen, L., 1994. Pathogenesis of measles virus infection: An hypothesis for altered immune responses. J. Infect. Dis. 170 (Suppl 1), S24–31.

Kimman, T., Bianchi, A., Wensvoort, G., Bruin, T., Meliefste, C., 1993. Cellular immune response to Hog Cholera Virus (HCV): T cell of immune pigs proliferate in vitro upon stimulation with live HCV, but the E1 envelop glycoprotein in not a major T-cell antigen. J. Virol. 67, 2922–2927.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Linné, T., Berg, M., Bergavall, A.C., Hjertner, B., Moreno López, J., 1992. The molecular biology of the porcine paramyxovirus LPMV. Vet. Microbiol. 33, 363–373.

McNeilly, F., Walker, I., Allan, G.M., Foster, J.C., Linné, T., Merza, M., Hernández, P., Kennedy, S., Adair, B., 1997. A comparative study on the use of virus and antibody detection techniques for the diagnosis of La Piedad Michoacan paramyxovirus (LPMV) infection in pigs. J. Vet. Diagn. Invest. 9, 3–9.

Moreno-López, J., Correa-Girón, P., Martínez, A., Ericsson, A., 1986. Characterization of a paramixovirus isolated from brain of a piglet in México. Arch. Virol. 91, 221–231.

Ramírez-Mendoza, H., Carreon, N.R., Mercado, G.C., Rodríguez, T.J., 1996. Hemoaglutinación e Inhibición de la hemoglutinación del paramixovirus porcino a través de la modificación de algunas variables que participan en la prueba. Vet. Mex. 27, 257–260.

Ramírez-Mendoza, H., Hernández-Jauregui, P., Reyes-Leyva, J., Zenteno, E., Moreno-Lopez, J., Kennedy, S., 1997. Lessions in the reproductive tract of boars experimentally infected with the porcine rubulavirus. J. Comp. Path. 117, 237–252.

Reyes-Leyva, J., Hernández-Jáuregui, P., Montaño, L.F., Zenteno, E., 1993. The porcine paramixovirus LPM specifically recognizes sialyl (α2,3) lactose-containing structures. Arch. Virol. 133, 195–200.

Reyes-Leyva, J., Espinosa, B., Hernandez, J., Zenteno, R., Vallejo, V., Hernandez-Jauregui, P., Zenteno, E., 1997. NeuAcα2,3Gal-Glycoconjugate expression determines cell susceptibility to the porcine rubulavirus LPMV. Comp. Biochem. Physiol. 118B, 327–332.

Rima, B., Alexander, D.J., Billeter, M.A., Collins, P.L., Kingsbury, D.J., Lipkind, M.A., Nagai, Y., Orvell, C., Pringle C.R., ter Meulen V., 1995. Family Paramyxoviridae. In: Murphy, F.A., Fauquet, C.M., Bishop, D.H.L. (Eds.), Virus Taxonomy. Classification and Nomenclature of Viruses, Springer, Wien, NY, pp 268–274.

Scott, P., Kaufmann, S.H.E., 1991 The role of T subsets and cytokines in the regulation of infection. Immunol. Today 160, 612–617.

Stephano, H.A., Gay, M., Ramírez, T.C., 1988. Encephalomyelitis, reproductive failure and corneal opacity (Blue Eye) in pigs, associate with a paramixovirus infection. Vet. Rec. 122, 6–10.

Stephano, H.A., 1994. Blue Eye diseases. In: Leman, A.D., Straw, B.F., Mengeling, W.L., Allaire, A.D., Taylor, D.J. (Eds.), Swine Diseases. Iowa State University press, Ames, IA, pp. 237–241.

Summerfield, A., Rhiza, H.J., Saalmueller, A., 1996. Functional characterisation of porcine $CD4^+CD8^+$ extrathymic T lymphocytes. Cellular Immunol. 168, 291–296.

Sundqvist, A., Berg, M., Hernández-Jáuregui, P., Linné, T., Moreno-López, J., 1990. The structural proteins of porcine paramixovirus (LPMV). J. Gen. Virol. 71, 609–613.

Sundqvist, A., Berg, M., Moreno-López, J., Linné, T., 1992. The hemagglutinin–neuraminidase glycoprotein of the porcine paramyxovirus LPM: Comparation with others paramyxovirus revealed the closest relationship to Simian virus 5 and Mumps virus. Arch. Virol. 122, 331–340.

Svenda, M., Berg, M., Moreno-López, J., Linné, T., 1997. Analysis of the large (L) protein gene of the porcine rubulavirus LPMV: Identification of possible functional domains. Virus Res., in press.

Tripp, R.A., Hou, S., NcMickle, A., Hiuston, J., Doherty, P.C., 1995. Recruitment and proliferation of $Cd8^+$ T cells in respiratory virus infections. J. Immunol. 154, 6013–6021.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.

Zenteno, R., Hernández, J., Espinosa, B., Hernandez-Jauregui, P., Reyes, J., Zenteno, E., 1998. Secondary structure prediction of the hemagglutinin-neuraminidase from a porcine rubulavirus. Arch. Virol. 143, 333–352.

Zinkernagel, R.M., Bachmann, M.F., Kundis, T.E., Ochen, S., Pirchet, H., Hengartner, H., 1996. On immunological memory. Ann. Rev. Immunol. 14, 333–368.

Zuckermann, F.A., Husmann, R.J., 1996. Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. Immunology 87, 500–512.

## CAPITULO II. Características de la respuesta Inmune celular del cerdo.

### Introducción

En este apartado se describen las características más importantes de la respuesta Inmune celular del cerdo, enfocándose básicamente en la participación de los linfocitos CD4+CD8+ en la respuesta inmune del cerdo. Los linfocitos T se dividen en cooperadores (linfocitos $CD4^+$) y citotóxicos ($CD8^+$). Los primeros regulan la respuesta inmune con la producción de citocinas como la IL-2, el INF$\gamma$, la IL-4 y la IL-10, los segundos son citotóxicos con restricción por el MHC de clase I (102). Ambos linfocitos maduran en el timo y cuando salen pierden el marcador de superficie CD1 que los identifica como linfocitos inmaduros y conservan el CD2 a diferencia de otros tipos celulares. Durante el proceso de maduración, otros marcadores de superficie se expresan en los linfocitos, tal como el receptor de linfocitos T (TcR$\alpha\beta$ y TcR$\gamma\delta$), las moléculas CD4 y CD8, entre otros (30). En las primeras etapas de maduración de los linfocitos se observa la expresión simultánea de las moléculas CD4 y CD8, pero antes de salir del timo y poblar los órganos linfoides secundarios se pierde una de las moléculas, y así en la sangre periférica bajo condiciones normales, no se observan altos porcentajes de linfocitos con doble marca en las células humanas y murinas. El cerdo expresa altos porcentajes de linfocitos dobles positivos en sangre periférica (entre 10 y 60%), así como en órganos linfoides secundarios en condiciones normales (33, 103).

Los linfocitos CD4+CD8+ son células que han adquirido el marcador CD8 después de su sensibilización y lo retienen para convertirse en linfocitos pequeños (36). Los linfocitos CD4+CD8+ están restringidos por el MHC-II, lo cual indica que el CD4 es la molécula responsable de la respuesta, sin descartar la posibilidad de algún papel biológico del CD8, el cual hasta la fecha no se ha identificado (41). Los linfocitos CD4+CD8+ expresan el antígeno CD29 (36), marcador que se asocia con la memoria inmunológica (46). La mayoría de los linfocitos CD4+CD8+

expresan altos niveles de CD29 ($CD29^{Alto}$) mientras que la mayoría de los linfocitos CD4+CD8- expresan bajos niveles de CD29 ($CD29^{Bajo}$). Estos resultados muestran la participación de las células dobles positivas en la memoria inmunológica del cerdo (36). En esta parte del proyecto se analizó la participación de los linfocitos CD4+CD8- y CD4+CD8+ durante la respuesta inmune de cerdos infectados experimentalmente con el rubulavirus porcino. Los resultados se presentan en el trabajo: Comparative evaluation of the CD4+CD8+ and CD4+CD8- lymphocytes in the immune response to porcine rubulavirus. En prepración para Veterinary Immunology and Immunopathology.

**Resultados y discusión**

Las CMN de los cerdos infectados experimentalmente proliferan en presencia del rubulavirus porcino (43). El análisis del fenotipo de las células que proliferan mostró linfoblastos CD4+CD8- y CD4+CD8+ sugiriendo la participación activa de estos linfocitos durante la respuesta inmune al rubulavirus porcino. Los resultados mostraron que los linfocitos CD4+CD8- y CD4+CD8+ proliferan después del estimulo antigénico. Sin embargo, su participación depende del momento en el cual se evaluó la proliferación. Cuando el análisis se realizó en las primeras semanas de infección únicamente los linfocitos CD4+CD8- proliferaron en respuesta al estimulo antigénico. Por el contrario, cuando la proliferación se analizó en la 8ª semana de infección las únicas células que proliferaron en presencia del antígeno fueron los linfocitos CD4+CD8+. Esto supone que los linfocitos CD4+CD8- son las células que participan en la respuesta aguda al rubulavirus, mientras que linfocitos CD4+CD8+ son células efectoras de memoria que se encargan de la respuesta tardía. Lo anterior se apoya en el hecho de que después de estimular linfocitos CD4+CD8- con el rubulavirus y determinar el fenotipo de estas células se observó que muchas de ellas se convirtieron en linfocitos CD4+CD8+. En los linfocitos CD4+CD8+ no se observaron cambios en el fenotipo sugiriendo que los linfocitos CD4+CD8- son la fuente principal de las

células efectoras de memoria CD4+CD8+, y por lo tanto se incrementan *in vivo* a partir de la 4ª semana de infección. Los linfocitos CD4+CD8+ que se observan durante la respuesta tardía al rubulavirus porcino son el resultado de la estimulación antígeno-específica de linfocitos CD4+CD8-, ya que en presencia de PHA no se observó un incremento en las células CD4+CD8+ y que los linfocitos CD4+CD8- de cerdos sin infectar no generan linfocitos CD4+CD8+. También se ha evaluado la participación de los linfocitos CD4+CD8- y CD4+CD8+ durante la respuesta inmune del cerdo en animales inmunizados con el virus de la enfermedad de Aujeszky (36, 41). Por un lado, los resultados indican que los linfocitos CD4+CD8- y CD4+CD8+ tienen la misma participación en la respuesta *in vitro* al virus inactivado de la enfermedad de Aujeszky (36). Por otro lado, Summerfield describe que únicamente los linfocitos CD4+CD8+ son capaces de proliferar frente al virus. Las diferencias en el haplotipo de los cerdos, el protocolo de inmunización y el tiempo en el cual se tomaron las muestras explican esas discrepancias. De acuerdo a nuestros resultados, proponemos que las diferencias de ambos autores obedecen a las propiedades intrínsecas de cada una de estas poblaciones durante la respuesta inmune del cerdo, ya que los linfocitos CD4+CD8- son las encargadas de la respuesta aguda y los linfocitos CD4+CD8+ se encargan de la respuesta secundaria.

En el humano, los linfocitos T de sangre periférica generalmente se encuentran dentro de uno de los siguientes estados: virgen, memoria o efectora. Una célula virgen es un precursor en circulación que aún no encuentra el antígeno especifico. Una célula de memoria deriva de una célula virgen después que esta encuentra su antígeno en el contexto de una CPA, pero posteriormente regresa a un estado de reposo listo para responder en una respuesta secundaria (104). En el cerdo se ha demostrado que la mayoría de los linfocitos CD4+CD8- expresan marcadores que las asocian con un estado de células vírgenes (36). Mientras que los linfocitos CD4+CD8+ son células en reposo que expresan marcadores de memoria (36, 46). Además, se ha descrito que las células de memoria se presentan en una frecuencia más alta que las células virgen y son células de vida

larga, las cuales pueden persistir por meses o años (55). En el caso del cerdo, los linfocitos CD4+CD8+ incrementan con la edad del animal (36, 37) y en procesos infecciosos (43). Sin embargo, tanto las células efectoras como las de memoria son células con experiencia antigénica, que llevan a cabo las funciones especializadas de las células T como secretar citocinas para la cooperación con las células B o la eliminación de células infectadas por virus (55). En este sentido, algunos autores han descrito a los linfocitos CD4+CD8+ como linfocitos CD4+CD8- que se activan y expresan el marcador CD8, pero suponen que no son más que linfocitos CD4+CD8- activados (34). Lo anterior de ninguna manera contradice lo que otros autores han supuesto. Por el contrario, nosotros hemos observado que los linfocitos CD4+CD8- al participar en la respuesta temprana al virus podrían ser considerados como linfocitos activados, muchos de los cuales expresan el marcador CD8 y se convierten en linfocitos CD4+CD8+ que posteriormente llevaran a cabo las funciones de una célula de memoria.

Las células efectoras se definen como células de vida corta que se generan de una célula de memoria o una célula virgen después de la estimulación. Generalmente estas son células terminales y mueren después de la estimulación. Los linfocitos CD4+CD8- vírgenes producen únicamente IL-2 en una estimulación inicial, mientras que las células de memoria tienen la capacidad de producir citocinas tipo Th1 (INF-$\gamma$ y TNF-$\beta$) y Th2 (IL-4 e IL-5) (104). Originalmente la IL-10 se describió como una citocina tipo Th2 que inhibía la síntesis de citocinas, particularmente el INF-$\gamma$ de las respuestas Th1 (105). Aunque la IL-10 puede tener algunos efectos directos en las células T, la principal actividad inhibitoria se ha asociado con las funciones de macrófagos y células presentadoras de antígeno (106). En humanos la IL-10 regula negativamente la respuesta inmune de manera que no discrimina entre Th1 o Th2. De hecho, la IL-10 regula negativamente la síntesis de citocinas dependientes de CPA por células humanas Th0, Th1 y Th2 (107). De esta manera se ha propuesto que a diferencia de la regulación clásica de Th1/Th2, la IL-10 representa un agente anti-inflamatorio cuya función es modular negativamente las respuestas Th1 y Th2 (107).

La IL-10 también presenta propiedades proliferativas, activadoras y quimiotácticas sobre varios tipos de linfocitos T. En particular la IL-10 actúa como un quimioatrayente de linfocitos CD8 (108) y aumenta la diferenciación, crecimiento y actividad citoxicidad de los linfocitos CD8 (109) y NK (110). Sobre células B, induce la expresión de moléculas de clase II, aumenta el crecimiento y diferenciación y las rescata de la apoptosis de los centros germinales (111). Nuestros resultados indican que los linfocitos CD4+CD8+ son una fuente importante de IL-10, más importante que los linfocitos CD4+CD8-. Estos resultados, por un lado, muestran que además de las diferencias fenotípicas y biológicas, estas células producen diferentes perfiles de citocinas. Así, los linfocitos CD4+CD8+ además de cooperar con las células para la producción de anticuerpos, son capaces de regulan las respuestas inflamatorias y favorecen la respuesta citotóxica durante las infecciones virales.

En resumen, este estudio demuestra que el rubulavirus porcino estimula la respuesta de linfocitos CD4+CD8- y CD4+CD8+. Las diferencias biológicas entre las dos poblaciones durante la respuesta inmune permite establecer que los linfocitos CD4+CD8+ son células importantes en la regulación de la respuesta inmune al rubulavirus porcino.

# Comparative evaluation of the CD4+CD8+ and CD4+CD8- lymphocytes in the immune response to porcine rubulavirus

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**Abstract**

The porcine immune system is unique in the expression of CD4+CD8+ (double-positive) lymphocytes. These cells have been associated with immunological memory due to their gradual increase with age, the expression of memory phenotype and their ability to respond to recall viral antigen. This work analyzes the biological function of CD4+CD8- and CD4+CD8+ lymphocytes in the immune response to the porcine rubulavirus. CD4+CD8- cells isolated from porcine rubulavirus infected pigs after three weeks of post-infection proliferated in response to homologous virus and generated lymphoblasts which were predominantly of the CD4+CD8+ phenotype; stimulation of CD4+CD8- cells with mitogen did not switch the phenotype. CD4+CD8- lymphocytes isolated after 10 weeks of infection proliferated in response to homologous virus and PHA but did not change their phenotype. CD4+CD8+ lymphocytes responded to PHA or viral antigen but did not change their phenotype The cytokine profile of both lymphocyte populations stimulated with PMA/Ionomicine showed the presence of IL-2 and IL-10 transcripts, but their quantitation demonstrated that double positive cells express mainly IL-10 whereas CD4+CD8- lymphocytes express basically IL-2. Our results show that CD4+CD8- lymphocytes participating in the early phase of the porcine rubulavirus infection, regulate memory phenotype expression.

Key words: porcine rubulavirus; reverse transcriptase-polymerase chain reaction, RT-PCR; T-lymphocytes, CD4+CD8+ double-positive T cells, viral immune response; swine.

## 1. Introduction

The porcine rubulavirus is responsible for the pigs´ blue eye disease which cause neurological, respiratory, and reproductive disorders (Moreno-Lopez et al., 1986; Stephano et al., 1998; Ramirez et al., 1997). The humoral response of experimentally infected pigs has shown that the main antigen is the virus´ Hemagglutinin-Neuraminidase (HN) (Hernandez et al., 1998) which recognizes host cells throughout sialyl$\alpha$2,3lactose-rich molecules (Reyes-Leyva et al., 1993; Reyes-Leyva et al., 1999; Vallejo et al., 2000). We have previously shown that peripheral blood mononuclear cells (PBMC) of experimentally infected pigs proliferate in the third week post-infection in the presence of the viral antigen and that this proliferative lymphoblasts possess the $CD4^+CD8^-$ and $CD4^+CD8^+$ (double-positive; DP) phenotype. Although the participation of the former lymphocytes in the immune response to porcine rubulavirus was anticipated, that of the $CD4^+CD8^+$ cells was unclear (Hernandez et al., 1998). Nevertheless, *in vitro* experiments have shown that DP cells can be generated from antigen-stimulated $CD4^+CD8^-$ lymphocytes (Zuckermann and Husmann, 1996), and that they proliferate not only to homologous-virus stimulated PBMC cultures (Pescovitz et al., 1994) but also to some parasites (Ivanoska et al., 1990).

The amount of $CD4^+CD8^+$ lymphocytes varies with age. In one month-old piglets they represent 1% of all peripheral blood T-cells while in three year-old pigs they represent up to 60% (Pescovitz et al , 1985; Saalmuller et al., 1989; Yang and

Parkhouse, 1996; Zuckermann and Husmann 1996). This double positive cells express CD3 (Yang and Parkhouse, 1996), class-II histocompatibility antigens (Saalmuller et al., 1991) and they do not express CD1 (Saalmuller et al., 1989). $CD4^+CD8^+$ lymphocytes express a low density of CD8 ($CD8^{low}$) (Saalmuller et al., 1989), and it has been suggested that the CD8 molecule in this cell population is made of homodimers of the $\alpha$ chain (Yang and Parkhouse, 1997; Zuckermann et al., 1998). Phenotype analysis of $CD4^+CD8^+$ cell surface molecules has demonstrated that 75% of this cells express the $\alpha4/\beta4$ integrin in great densities ($CD29^{high}$), a molecule related to immune memory in human lymphocytes (Morimoto et al., 1995), in marked contrast to 25% of the $CD4^+CD8^-$ lymphocytes which express it in a low density (Zuckermann and Husmann, 1996). It has also been proposed that, in another virus-induced disease, Aujeszky disease, double positive T cells cooperate with B cells in the antibody-producing response (Ober et al., 1998). Therefore, the aim of this work was to determine if the role of the two well characterized porcine $CD4^+CD8^+$ and $CD4^+CD8^-$ cell populations in the immune response to porcine rubulavirus is complementary or dissimilar.

## 2. Material and Methods

### *2.1. Animals*

Crossbred domestic swine (Landrace X, Duroc) adults pigs (>7 months) were obtained from a commercial farm. Animals were certified to be free of most swine diseases including porcine rubulavirus. Pigs were experimental infected by intranasal administration of the porcine rubulavirus ($10^4$ $TCID_{50}$/ml) as previously reported (Hernandez et al., 1998) and sacrificed at the end of the experiment under the procedures described by the office of laboratory animal care which are based on federal guidelines.

### *2.2. Antibodies*

The following porcine-specific mouse monoclonal antibodies (mAb) were gently provided by Dr M. Petscovitz (Indiana University, Indianapolis USA): anti-CD4 (clone 74.12.4, IgG2b), anti-CD8 (clone 76.2.11, IgG2a) and anti-monocyte (clone CAM36A, IgG1). The specificity of these mAb has been described previously (Pescovitz et al., 1984). Fluorescein isothiocyanate (FITC)-labeled rat anti-mouse IgG2b and phycoerythrin (PE)-labeled rat anti-mouse IgG2a were from Serotec Ltd (Oxford, England).

*2.3. Two color analysis*

For double cytofluorometric analysis, cells were incubated with 10µl of the anti-CD8 and 10 µl of the anti-CD4 monoclonal antibodies for 15 minutes at 4°C followed by incubation with both FITC- and PE-conjugated isotype-specific rat antibodies. All incubations were carried out at 4°C during 15 min. Cells were washed with 0.1 M PBS, pH 7.2, containing 0.3% bovine serum albumin (BSA). Stained cells were analyzed by cytofluorometry (FACS Excalibur Becton & Dickinson, Mountain View, CA USA).

*2.4. Fluorescent-activated cell sorting of T cells subpopulations*

CD4+CD8- and CD4+CD8+ lymphocytes were isolated by two-color FACS. Eppendorf tubes containing $10^7$ of glass-adherent cell-depleted peripheral blood mononuclear cells in sterile PBS/BSA, were incubated with anti-CD4 and anti-CD8. After a 15 min incubation on ice, cells were washed with PBS and reacted with FITC-labeled rat anti-mouse IgG2b, and with PE-labeled rat anti-mouse IgG2a for 45 minutes. After a final wash, cells were suspended to $10^6$/ml in PBS and sorted into $CD4^+CD8^-$ and $CD4^+CD8^+$ populations. There was no crossover between the two sorted fractions, as checked by cytofluorometric analysis of each sorted population. Stained unsorted and unstained cells served as controls of the possible effect of the treatment on the response to stimulation.

*2.5 Isolation of Antigen Presenting Cells*

A single cell suspension of PBMC was stained with 100µl of a mouse anti-pig monocytes monoclonal antibody at 4°C during 15 min. Cells were washed with 0.1 M PBS, pH 7.2, and 0.3% BSA and incubated with a sheep anti-mouse immunoglobulin-coated magnetic particles (MACS, Miltenyi Biotec Inc., Sunnyvale, CA, USA) for 15 minutes at 4°C. The purity of the sorted cells was confirmed by flow cytometry (>96%).

*2.6 Lymphoproliferation assay*

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples by Ficoll-Hypaque (Pharmacia) gradient centrifugation. Fluorescent-activated cell sorting of T cell subpopulations from healthy and infected pigs at different times after infection were cultured at $2.5 \times 10^5$/well in sterile 96 well round bottom plates in a 200 µl volume of HEPES-buffered RPMI-1640 culture medium, supplemented with $2 \times 10^{-5}$ M 2-mercaptoethanol, 2mM sodium pyruvate, 2mM L-glutamine, 1 µg/ml of gentamycin, and 10% of fetal calf serum (Sigma Chemicals, St. Louis, MO). Lymphocytes were stimulated with 10 µg/ml of viral antigen for 5 days in a humidified incubator with 5% $CO_2$ at 37° in the presence of $0.25 \times 10^5$ autologous antigen presenting cells (APC). Viral antigen consisted of supernatants of infected pig kidney (PK) cells ultracentrifuged at 100,000 g for 4 h at 4°C, filtered

through 0.45 µm membranes, aliquoted and stored at -70°C until use. Measles virus stock was grown in Vero cells for 4 days in MEM medium supplemented with 2% fetal calf serum and 50 µg/ml of gentamycin; the supernatant of these cultures was heat inactivated and was used at 1:100 dilution as control antigen. Cell cultures without stimulation were used as negative control. Positive response controls consisted of stimulation with the T-cell mitogen, phytohemagglutinin (PHA) (8 µg/ml) (Gibco). All experiments were performed in triplicate. Cell proliferation was measured by the addition of 50 µl/well of culture medium containing 1 µCi of $^{3}$Hthymidine (New England Nuclear, specific activity 52 Ci/mmol) during the last 16-18 h of culture. Cells were harvested onto glass fiber filter paper with a cell harvester, and incorporated radioactivity was measured in a Beckman LS6000 SE-counter (Beckman, Fullerton CA.). Results are expressed as mean counts per minute (c.p.m.) ± standard deviation of triplicate cultures.

*2.7 RNA extraction*

Total RNA was extracted from cells pellets using TRIzol (Gibco) according to the manufacturer's protocol. RNA was suspended in 20µl of ultra-pure water containing 0.02% (w/v) of diethyl pyrocarbonate (Sigma). Total RNA was quantified using a spectrophotometer at $O.D._{260}$ and the purity was assessed by determining the $O.D._{260}/O.D._{280}$ ratio. All samples had $O.D._{260}/O.D._{280}$ ratios above 1.9

*2.8 RT-PCR assay*

Reverse transcription and PCR reactions were performed in a Touchdown, Hybaid Thermal Cycler (Perkin-Elmer) thermocycler. Reverse transcription of RNA into cDNA was performed in a 10 μl volume of a mixture containing: 1 μg of sample RNA; 0.072 μg of oligo$(dT)_{12-18}$; 4.8mM of deoxynucleotide triphosphate (dNTP) mix of the four dNTPs; 1x reverse transcriptase buffer (20mM Tris-HCl, pH 8.4, 50nM KCl); 6.0mM of $MgCl_2$; 8mM of DTT; 9.6 U of RNasin inhibitor; and 48 U of M-MLV reverse transcriptase (Gibco BRL). The reaction mixture was incubated at 42° C for 60 min. The oligonucleotide primers used for the detection of cDNA specific to porcine interleukines IL-2, IL-4, IL-10, IFN-γ and β-actin as a constitutively expressed 'housekeeping' gene, have been previously reported (Dozois et al., 1997). The PCR contained 3 μl of first strand cDNA reaction mixture, 10 pM of suitable primer set, and 1.25 U/15 μl of reaction final volume for each primer set of Taq DNA polymerase (Gibco BRL). After an initial denaturation step at 94°C for 5 min, cycling conditions were 94°C for 50 s, 54°C for 50 s, 72°C for 50 s and finally 7 min at 72°C. PCR reaction sample (5 μl, each) were migrated on 1.2% TBE agarose gels. Gels were stained with ethydium bromide.

*2.9 Densitometric semi-quantification of PCR products*

To compare the relative mRNA expression level from each sample, the PCR

products of the different cytokines tested were quantified comparing their intensity value (Imaging and analysis system UVP's GDS-8000 PC-based system) as determined by densitometric analysis, with that of the transcripts of the β-actin gen which were considered as the reference value. The results are presented as a relative intensity ratio of the cytokine RT-PCR product over the corresponding β-actin RT-PCR product, and the differences in expression between lymphocyte subpopulations is expressed in percentage.

## 3. Results

### *3.1 $CD4^+CD8^-$ and $CD4^+CD8^+$ lymphocytes in response to porcine rubulavirus*

Cytofluorometric analysis of sorted $CD4^+CD8^-$ and $CD4^+CD8^+$ lymphocytes showed a purity greater than 94% in all experiments. Staining of the sorted cell subpopulations with specific monoclonal antibodies did not impair their response to viral antigen or mitogen as compared to untreated cells in the 5-day proliferation assay (data no show). The proliferation assays were performed with sorted cell populations obtained from pigs experimentally infected with porcine rubulavirus (PRv). $CD4^+CD8^-$ lymphocytes isolated from PRv-infected pigs in the 3rd week post-infection proliferated in response to viral antigen ($p<0.001$; figure 1a), as opposed to $CD4^+CD8^+$ lymphocytes which didn't respond ($p=0.1$; figure 1b). When the proliferative response was evaluated 10 weeks post-infection, $CD4^+CD8^-$ lymphocytes did not respond ($p=0\ 1$; figure 1c), whereas the $CD4^+CD8^+$ cells responded ($p<0.001$; figure 1d). The cellular response was a recall response, demonstrated by the fact that sorted lymphocytes obtained from a porcine rubulavirus-naive pig did not respond to stimulation with PRv antigen. The specificity of the response was demonstrated by the lack of proliferative response with Measles virus. $CD4^+CD8^+$ and $CD4^+CD8^-$ cell populations proliferated in response to PHA (figure 2a and b), however, the magnitude of the proliferative response of the $CD4^+CD8^+$ cells was 56% lower ($p=0.01$) than that of $CD4^+CD8^-$ cells.

*3.2 $CD4^+CD8^+$ cells are generated in vitro by $CD4^+CD8^-$ antigen-specific stimulation*

To determine if PRv-specific $CD4^+CD8^+$ lymphocytes could be generated *in vitro*, $CD4^+CD8^-$ lymphocytes isolated from three weeks PRv-post-infected pigs were stimulated with viral antigen in the presence of antigen presenting cells, for 5 days. Populations of resting (small lymphocytes) and activated (lymphoblasts) cells within a culture were separated by their characteristic light scatter profile and analyzed. When compared with control assays the percentage of small lymphocytes $CD4^+CD8^+$ generated from $CD4^+CD8^-$ stimulated with PHA remained constant (Figure 3a,b), whereas this cell population increased when they were stimulated with PRv (Figure 3c). The percentage of lymphoblasts increased from <5% in unstimulated cultures to 12-20% in PHA or Paramyxovirus-stimulated cultures; interestingly, approximately 50% of the latter and less than 3% of the former had a $CD4^+CD8^+$ phenotype (Figure 3d-f). $CD4^+CD8^+$ lymphocytes stimulated with PRv or PHA did not change their phenotype.

*3.3 $CD4^+CD8^-$ and $CD4^+CD8^+$ lymphocytes in the regulation of porcine immune response*

The cytokine profile of sorted porcine $CD4^+CD8^-$ and $CD4^+CD8^+$ lymphocytes obtained after ten weeks of infection showed transcripts for IL-2 and IL-10 (Fig. 4a). The quantitative IIR (see material and methods) for β-actin was 245

in both cell populations. The IL-2 IIR for $CD4^+CD8^-$ lymphocytes was 148 against 128 for $CD4^+CD8^+$ lymphocytes; the IIR for IL-10 was 98 for the former and 200 for the latter (Figure 4b). We did not observe expression of IL-4 and IFN-$\gamma$ on either $CD4^+CD8^-$ or $CD4^+CD8^+$ lymphocytes.

## 4. Discussion

The analysis of different porcine T cell lymphocytes has shown many interesting immunological features, specially in the $CD4^+CD8^-$ and $CD4^+CD8^+$ subpopulations Both populations although similar in many ways (cell surface CD markers, MHC-II restriction) (Yang and Parkhouse, 1996; 1997; Saalmuller et al., 1991;1989; Zuckermann et al., 1998) show some differences namely the expression of memory markers such as $\alpha 4/\beta 4$ or CD29 integrins (Zuckermann and Husmann, 1996). Seventy five percent of $CD4^+CD8^+$ lymphocytes are $CD29^{high}$ as opposed to only 25% of $CD4^+CD8^-$ cells. In this work we tried to establish the role of the $CD4^+CD8^-$ and $CD4^+CD8^+$ lymphocytes in the immune response against the porcine rubulavirus. Our results showed that besides the phenotype there are important immunobiological differences between both cell populations. CD4+CD8- lymphocytes proliferated in a secondary response manner in the first weeks of infection whereas the same behavior was seen in CD4+CD8+ cells after ten weeks of infection had elapsed. Similar results have been observed in the Aujezsky disease where CD4+CD8- lymphocytes participate in the acute response (Zuckermann and Husmann, 1996) and the CD4+CD8+ cells engage in memory immune responses. The latter are $CD29^{high}$, a phenotype usually associated with a memory/effector activity, as opposed to the former which are $CD29^{low}$ (Zuckermann and Husmann, 1996; Summerfield et al., 1996).

The association between the appearance of CD4+CD8+ lymphocytes and

age (Saalmuller et al., 1989), or their presence after an infection (Hernández et al., 1998) assumes the participation of antigen stimulation in the induction of these cells. Our results show that lectins such as Con-A or PHA didn't modify the phenotype of CD4+CD8- and CD4+CD8+ lymphocytes; but when CD4+CD8- cells were stimulated with the virus we observed lymphoblasts with the CD4+CD8+ phenotype, in a similar manner to previous results (Zuckermann and Husmann, 1996). It has been observed that human CD4+CD8+ lymphocytes are generated from CD4+CD8- cells stimulated with mitogen or alloantigen in the presence of exogenous IL-4, which is fundamental to keep the stability of the newly formed CD4+CD8+ cells (Paliard et al., 1988; Brod et al., 1990). Porcine lymphocytes do not require such interleukin although we can not exclude the presence in vivo of such interleukin as a consequence of the natural infection and the inflammatory process that ensues. It is possible that coexpression of CD4 and CD8 molecules in a memory cell enhances the avidity of the TCR with its target cell thus reinforcing the importance of intracellular signals for cellular activation (Bierer et al., 1989). This cascade of events would facilitate the activation of memory double positive cells in the presence of low antigen concentrations.

One of the main functions of memory cells is to rapidly respond to a new viral encounter secreting cytokines (Dutton et al., 1998), either proinflammatory (Th1 profile- IFN-y, TNF-b, IL-2) or inducers of antiparasite and humoral immune response (Th2 profile- IL-4, IL-5, IL-9, IL-10, IL-13) (Mosmann et al., 1986). Our results showed that the main source of IL-10 were the $CD4^{+}CD8^{+}$ lymphocytes. This cytokine originally considered as an inhibitor of Th1 cytokines acts upon T

cells, macrophages, and antigen presenting cells (Fiorentino et al., 1989; Moore et al., 1993). Nowadays IL-10 is seen as an anti-inflammatory agent in charge of the negative feedback of Th1 and Th2 responses (Muraille and Leo 1998). Its action upon immune T cells include chemoattractant activity for CD8 cells (Jinquan et al., 1993), and enhancer of the growth, differentiation and cytotoxic activity of CD8 and NK cells (Chen and Zlotnik 1991; Carson et al., 1995). It also induces the expression of MHC class II molecules on B cells and enhances their growth and differentiation on top of rescuing them from apoptosis in the germinal centers (Levy and Brouet, 1994). Taken together, these results show that $CD4^+CD8^+$ are memory cells (Zuckermann, 1999) that indirectly participate in antibody production (Ober et al., 1998), regulate inflammatory events and favour the cytotoxic response in rubulavirus infection. In summary our work shows that the porcine rubulavirus stimulates the response of $CD4^+CD8^-$ lymphocytes which secrete IL-2 and that this cell population generates specific memory $CD4^+CD8^+$ lymphocytes which secrete IL-10, thus regulating, both cell populations and the immune response against the virus.

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## References


Bierer B.E, Sleckman B. P., Ratnofsky S. E., Burakoff S. J. (1989) The biologic roles of CD2, CD4, and CD8 in T-cell activation. *Annu. Rev. Immunol.* 7, 579-99.

Carson W.E., Lindemann M.J., Baiocchi R., et al. (1995) The functional characterization of interleukin-10 receptor expression on human natural killers cells. Blood 12, 3577-3582.

Chen W-F. & Zlotnik A. (1991) IL-10: a novel cytotoxic T cell differentiation factor. J. Immunol. 147, 528-536.

Dozois M. Ch , Oswald E., Gautier N., Sherhelon J., Fairbrother J., Oswald P.I. (1997) A reverse transcription-polymerase chain reaction method to analyzed procine cytokine gene expression. Vet. Immunol. Immunopathol. 58, 287-300.

Dutton R.W.,Bradley L.M., and Swain S.L. (1998) T cell memory. Annu. Rev. Immunol. 16, 201-223.

Fiorentino D F., Bond M.W., Mosmann T.R. (1989) Two types of mouse helper T cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J. Exp. Med. 170, 2081-2095.

Hernández J., Reyes-Leyva J., Zenteno R., Ramirez H., and Zenteno E. (1998) Immunity to porcine rubulavirus infection in adult pigs. Vet. Immunol. Immunopathol. 64, 367-381.

Ivanoska D, Cuperlovic K, Lunney JK. (1990) Peripheral blood mononuclear cells subsets during *Trichinella spiralis* infection in pigs. Res. Vet. Sci. 49, 92-97.

Jinquan T., Larse C.G., Gesser B., Matsushima K., Thestrup-Pedersen K. (1993) Human IL-10 is a chemoattractan for CD8+, T lymphocytes an inhibitor of IL-8-inducer CD4+ T lymphocyte migration. J. Immunol. 151, 4545-4557.

Levy Y. & Brouet J-C. (1994) Interleukin-10 prevents spontaneous death of germinal center B cells by induction of BCL-2 protein. J. Clin. Invest. 93, 424-430

Moore K.V., O'Garra A., de Waal Melafty R., Vieira P. and Mosmann T.R. (1993) Interleukin-10. Annu. Rev. Immunol. 11, 165-185.

Moreno-Lopez J., Correa-Giron P., Martinez A., Ericsson A. (1986) Characterization of a paramyxovirus isolated form the brain of a piglet in Mexico. Arch Virol 91, 221-231.

Morimoto C., Letvin N.L., Boyd AW., et al., (1985) The isolation and characterization of the human helper inducer T cell subset. J. Immunol. 134, 3762-3769.

Mosmann T.R., Cherwinski H.M., Bond M W., Geidlin M.A., Coofman 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, 2348-2354.

Muraille E.& Leo O. (1998) Revisiting the Th1/Th2 paradigm. Scand. J. Immunol. 47, 1-9.

Ober B., Summerfield A., Mattlinger C., Wiesmuller K., Jung G., Pfaff E., Saalmuller A., and Rziha H. (1998) Vaccine-induced, pseudorabies virus-specific, extrathymic CD4+CD8+ memory t-helper cells in swine. J Virol 72, 4866-4873.

Pescovitz M.D., Lunney J.K., & Sachs D.H. (1984) Preparation and characterization of monoclonal antibodies reactive with porcine PBL. J Immunol 133, 368-375.

Pescovitz M.D., Lunney J.K.& Sachs D.H. (1985) Murine anti-swine T4 and T8 monoclonal antibodies: distribution and effects on proliferative and cytotoxic T cells. J. Immunol. 134, 37-44.

Pescovitz M.C., Sakapoulos A. G., Gaddy J. A., Husmman R.J., Zuckermann F. (1994) Porcine peripheral blood CD4+/CD8+ dual expressing T-cells. Vet. Immunol. Immunopathol. 43, 53-62.

Ramirez M.H , Hernandez-Jauregui P., Reyes-leyva J., Zenteno E., Moreno-Lopez J., and Kennedy S. (1997) Lesions in the reproductive tract of boars experimentally infected with porcine rubulavirus. J. Comp. Pathol. 117, 237-252.

Reyes-Leyva J., Hernandez-Jauregui P., Montaño L.F., and Zenteno E. (1993) The porcine paramixovirus LPM specifically recognize sialyl (α2,3) lactose-contained structures. Arch. Virol. 133, 195-200.

Reyes-Leyva J., Espinosa B., Hernandez J., Zenteno R., Vallejo V., Hernandez-Jauregui P., and Zenteno E. (1997) NeuAcα2,3Gal glycocongugate expression determinates cell susceptibility to the porcine rubulavirus LPMV. Comp Biochem. Physiol. 118B, 327-332.

Saalmuller A, Hirt Werner, Reddehase M. (1989) Phenotypic discrimination between thymic and extrathymic CD4-CD8- and CD4+CD8+ porcine T lymphocytes. Eur. J. Immunol. 19, 2011-2016.

Saalmuller A., Hirt W., Redehasse M.J. (1991) Resting porcine T lymphocytes expressing class II major histocompatibility antigens. Immunobiology 183,102-114.

Stephano H.A., Gay G.H., and Ramires T.C. (1998) Encephalomyelitis, reproductive failure and corneal opacity (Blue eye) in pigs, associated with a paramixovirus infection. Vet. Rec. 122, 6-10.

Summerfield A., Rziha H., and Saalmueller A. (1996) Functional characterization of porcine CD4+CD8+ extrathymic T lymphocytes. Cellular Immunology 168, 291-296.

Vallejo V., Reyes-Leyva J., Hernández J., Ramirez H., Delannoy P., and Zenteno E. (2000) Differential expression of sialic acid on porcine organs during the maturation process. Comp. Biochem. Physiol B Biochem Mol Biol, 126: 415-24.

Yang H., and Parkhouse M. (1996) Phenotypic clasification of porcine lymphocytes subpopulation in blodd and lymphoid tissues. Immunology 89, 76-83.

Yang H., and Parkhouse M. (1997) Differential expression of CD8 epitopes amongst porcine CD8-positive functional lymphocytes subsets. Immunology 92, 45-52.

Zuckermann F., and Husmann R. (1996) Functional and phenotypic analysys of porcine peripheral blodd CD4/CD8 double-positive T cells. Immunology, 87, 500-512.

Zuckermann F., Pescovitz M., Aasted B., Dominguez J., Trebichavsky I., Novikov B., Valpotic I., Nielsen J., Arn S.. Sachs D., Lunney J., Boyd., Walker J., Lee R., Davis W., Barbosa I., and Saalmuller A. (1998) Report on the analyses of mAb

reactive with porcine CD8 for the second international swine CD workshop. Vet. Immunol. Immunopathol. 60, 291-303.

Zuckermann F. (1999) Extrathymic CD4/CD8 double-positive T cells. Vet Immunol Immunopathol 72, 55-66.

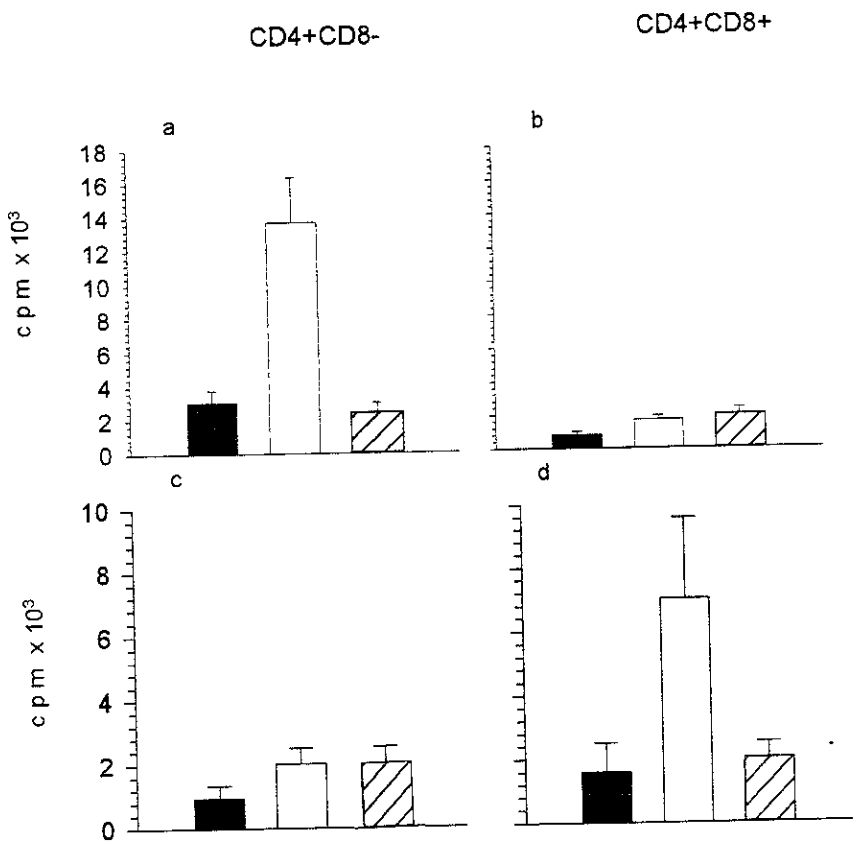


Figure 1. Proliferative response of CD4+CD8- and CD4+CD8+ lymphocytes to mitogen and viral antigen stimulation. FACS sorted CD4+CD8- or CD4+CD8+ (2.5 x $10^5$/well) isolated from naive or infected porcine rubulavirus swine, were stimulated with PRv (Clear bar) and Measles virus (Dash bar) for 5 days. Autologous APC (0.25 x $10^5$) were added to sorted cell populations. Proliferation was measured by $^3$Hthymidine incorporation during the last 18 h of culture. Values are expressed as mean±standard deviation of triplicate samples. *$p<0.05$ Control experiments were performed with non-stimulated cells (full bar).

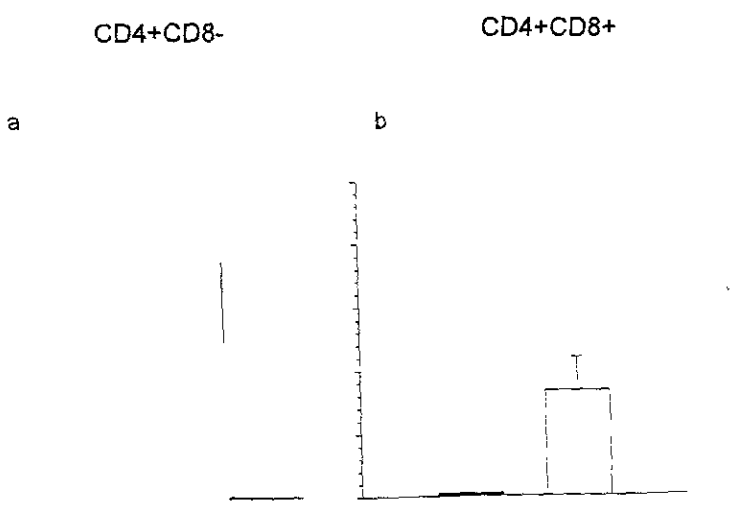


Figure 2. Proliferative response of CD4+CD8- and CD4+CD8+ lymphocytes to mitogen stimulation. FACS sorted CD4+CD8- or CD4+CD8+ lymphocytes (2.5 x $10^5$/well) isolated from infected porcine rubulavirus swine (n=5), were stimulated with 8µg/ml PHA for 3 days (Clear bar). Proliferation was measured by $^3$Hthymidine incorporation during the last 18 hr of culture. Values are expressed as mean±standard deviation of triplicate samples. *$p<0.05$.

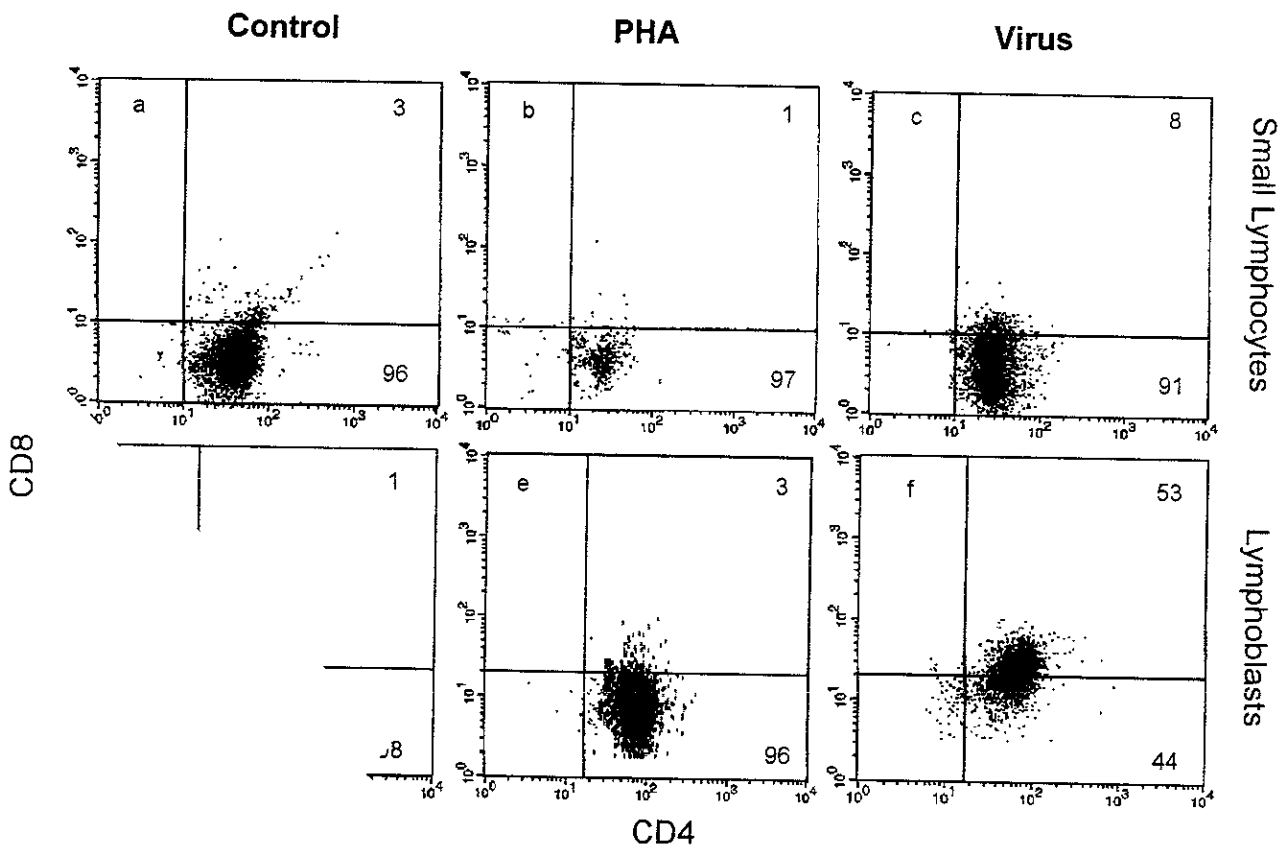


Figure 3. Two color cytofluorometric analysis of FACS-purified porcine lymphocytes after stimulation with PHA or recall viral antigen. The percentage of small lymphocytes $CD4^+CD8^+$ generated from $CD4^+CD8^-$ stimulated with PHA remained constant, whereas this cell population increased when they were stimulated with PRv (a-c) . Generation of $CD4^+CD8^+$ lymphoblasts from $CD4^+CD8^-$ increased in those cells stimulated with PRv (d-f).

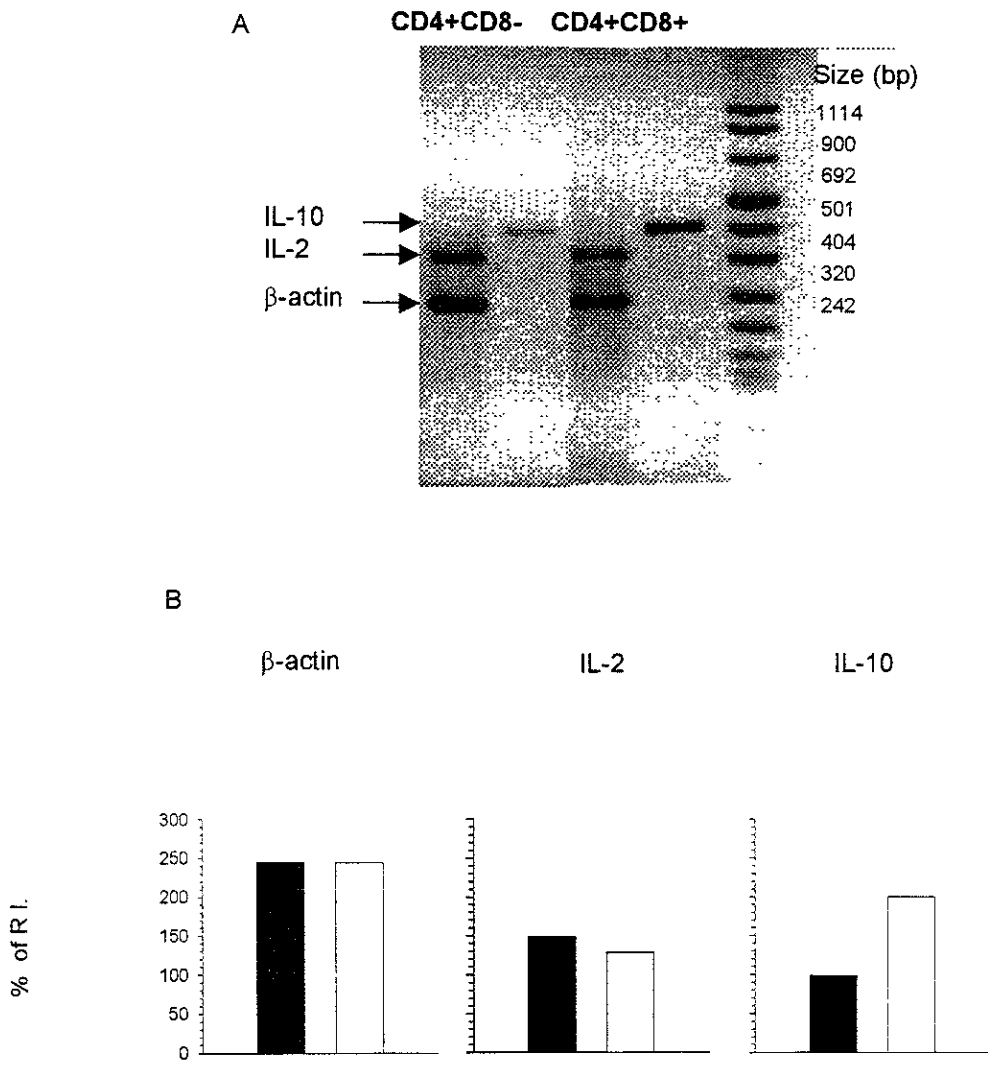


Figure 4. Cytokine expression on CD4+CD8- and CD4+CD8+ porcine lymphocytes. (A) CD4+CD8- and CD4+CD8+ porcine lymphocytes were stimulated with PMA/Ionomicin; the isolated RNA was RT-PCR to determine IL-2, IL-4, IL-10, IFN-γ and β-actin. The PCR products were migrated on 1.2% TBE agarose gels. (B) Densitometric semi-quantitative analysis of RT-PCR products of stimulated CD4+CD8- (full bar) or CD4+CD8+ lymphocytes (clear bar) was done as described in material and methods. We did not observed expression of IL-4 and IFN-γ on either $CD4^+CD8^-$ or $CD4^+CD8^+$ lymphocytes.

## Capítulo III. Expresión de estructuras O-glicosiladas en las subpoblaciones de linfocitos T porcinos.

### Introducción

Existen diferentes tipos de modificaciones postraduccionales que sufren las proteínas recién sintetizadas en los organismos vivos, la más común es la glicosilación (112). La glicosilación es especie y célula específica y está determinada por la unión covalente entre carbohidratos y el esqueleto de la proteína (113). La síntesis de glicoproteínas está codificada genéticamente y se realiza mediante enlaces de tipo O- (N-acetil-D-galactosamina unida a serina o treonina) y N-glicosídicos (N-acetil-D-glucosamina unida a asparagina) (112). Estos eventos se realizan de manera postraduccional en el retículo endoplásmico y son completadas en el aparato de Golgi con la adición de algunos carbohidratos como el ácido siálico. Las estructuras glicosiladas poseen una gran heterogeneidad que depende del tipo celular y del estado fisiológico de un organismo, tejido o célula en el cual la glicoproteína esta hecha (112, 114). En particular, el ácido N-acetil-neuramínico (ácido siálico, Neu5Ac) es una molécula presente en casi todas las células eucariontes que se une al carbono anomérico frecuentemente por enlaces de tipo $\alpha$2,3 o $\alpha$ 2,6 con galactosa y se ha demostrado que estas modificaciones son reguladas por el desarrollo de los grupos celulares, particularmente en aquellas poblaciones que participan en los diferentes procesos de respuesta inmune (115, 116). Las células nucleadas que se generan en la médula ósea presentan glicoproteínas O-glicosiladas del tipo mucina que contienen principalmente N-acetil-D-galactosamina (Gal-Nac) unida a los grupos hidroxilo de las serinas o treoninas. La adición de diferentes residuos de azúcares como galactosa (Gal) o ácido N-acetil-neuramínico (Neu5Ac) en las glicoproteínas O-glicosiladas de los linfocitos aparentemente depende del estado de activación o maduración de las células. Sin embargo, las estructuras tipo mucina pare ser

específico para cada tipo celular y los diferentes estados de activación (117) lo que sugiere la importancia de las estructuras O-glicosiladas en la fisiología celular.

El presente trabajo analiza la posibilidad de utilizar las lectinas PNA y de *Amaranthus leucocarpus* (ALL) para discriminar entre linfocitos porcino vírgenes y de memoria. La lectina PNA reconoce al -OH del C4 de galactosa y la lectina ALL, reconoce al –OH del C4 y al grupo $CH_3CO$ del C2 de la GalNAc en la secuencia Galß1,3GalNAc. En este trabajo se evalúan las principales características fenotípicas de estructuras O-glicosiladas de linfocitos T porcinos con la intención de identificar marcadores fenotípicos de poblaciones linfocitarias en diferentes estadios de maduración, memoria y efectoras, que podrían ser utilizadas como herramienta auxiliar para evaluar la efectividad de vacunas. Los resultados se presentan en el trabajo: Peanut agglutinin (PNA) and *Amaranthus leucocarpus* (ALL) lectins discriminate between memory and naive porcine lymphocytes. En preparación para Veterinary Immunology and Immunopathology.

**Resultados y discusión**

Los patrones de glicosilación cambian según el estado de maduración o de activación de célula. Esta propiedad permite utilizar las lectinas para distinguir los diferentes estados del proceso de diferenciación de los linfocitos T (119, 120). En este trabajo se utilizaron las lectinas PNA y ALL para caracterizar las subpoblaciones de linfocitos porcinos. Los resultados muestran que ambas lectinas reconocen más linfocitos CD4+CD8+ que CD4+CD8-. El reconocimiento de la lectina PNA se asoció con un fenotipo de memoria efectora que se incrementa después de la infección con el rubulavirus y responde proliferando en presencia del virus. Por el contrario, la lectina ALL se asoció con un fenotipo virgen que no se modifica después de la infección y no prolifera en presencia del virus. Estos resultados indican que la lectina PNA reconoce una población con características de una célula de memoria, mientras que ALL reconoce una célula con características de una célula virgen.

Existen al menos dos explicaciones para entender las diferencias en el reconocimiento de las lectinas PNA y ALL en los linfocitos porcinos. La lectina PNA reconoce específicamente la estructura Galß1 del disacárido Galß1,3GalNAc (74) y ALL reconoce GalNAc del disacárido Galß1,3GalNAc (120). Ambas estructuras se encuentran en oligosacáridos O-glicosilados unidas a las estructuras glicoproteícas en la superficie de las células. Las sialiltransferasas pueden adicionar ácido siálico a estos disacáridos dando como resultado la secuencia SAα2,3Galß1,3(SAα2,6)GalNAc (121). La presencia del ácido siálico evita el reconocimiento de la lectina PNA pero no de ALL. Para incrementar la unión de la lectina PNA a la superficie de las células es necesario que disminuya la expresión o actividad de las sialiltransferasas, o que los residuos de ácido siálico sean eliminados de las glicoproteínas nuevas o recicladas por neuraminidasas endógenas (122). La diferencia en la expresión de estructuras O-glicosiladas en los linfocitos CD4+CD8+ y CD4+CD8- puede deberse a la actividad de neuraminidasas endógenas que actúan en los linfocitos CD4+CD8- cuando se estimulan en presencia de virus para generar linfocitos CD4+CD8+, dejando estas células ricas en estructuras O-glicosiladas, como sucede en los linfocitos CD8 y CD4 de ratones infectados con el virus de la coriomeningitis linfocitaria (116). Las estructuras O-glicosiladas se han asociado con células T activadas *in vitro* (69), con linfocitos de efectores de memoria (116) y con linfocitos vírgenes (73). Esto sugiere que la expresión de las estructuras O-glicosiladas se puede asociar con diferentes estados de activación, y por lo tanto dentro de la población de linfocitos CD4+CD8+ podría existir una subpoblación con funciones de una célula efectora de memoria y otra que permanece en un estado de reposo o en transición. Las células de memoria pueden existir temporalmente como células efectoras, lo que correspondería a los linfocitos PNA+ALL-; una vez que la infección se ha controlado, muchas de las células efectoras de memoria pueden regresar a su estado de reposo y regular negativamente la unión a PNA (PNA-ALL+) y permanecer como células de memoria.

Se ha utilizado la expresión de algunas moléculas en la superficie de las células para distinguir entre linfocitos vírgenes y de memoria. En el humano, los linfocitos T efectores de memoria incrementan los niveles de expresión de diferentes moléculas de adhesión como el antígeno asociado a la función de los linfocitos-3 (LAF-3), LFA-1 y la integrina β1 (123). Las integrinas son moléculas muy conservadas entre diferentes especies, lo que se correlaciona con la reacción cruzada entre los anticuerpos contra vertebrados, invertebrados y hongos (124). El anticuerpo 4B4 reconoce la integrina humana β1 y leucocitos porcinos generando tinciones similares. Además, la inmunoprecitación de CMN marcadas identifica proteínas de peso similar (36). Estos resultados sugieren que este anticuerpo reconoce en el cerdo el homólogo CD29 del humano. Esta molécula se expresa en altos y bajos niveles en la superficie de los linfocitos porcinos y permite distinguir funcionalmente entre células virgen y efectoras de memoria. La expresión de esta molécula en los linfocitos porcinos CD4+CD8+ indica que 75% de ellos expresan altos niveles del CD29 ($CD29^{alto}$) lo cual supone que esta población contiene células efectoras de memoria (36). Nuestros resultados muestran que las células que son reconocidas por la lectina PNA expresan el fenotipo $CD29^{alto}$, mientras que las células reconocidas por la lectina ALL expresan el fenotipo $CD29^{bajo}$. Estos resultados suponen que la lectina PNA reconoce linfocitos con capacidad de memoria efectoras y ALL células vírgenes o en estado de reposo. El hecho de que la lectina PNA reconozca principalmente la población CD4+CD8+, la cual previamente hemos observado que se incrementa después de la infección con el rubulavirus porcino (43), podría explicar el incremento en los porcentajes de células PNA+$CD29^{alto}$ después de la infección.

Dentro de las glicoproteínas que reconoce la lectina PNA en timocitos de ratón se encuentran CD43, CD45 y CD8 (118). La molécula CD8 se expresa en forma de heterodímeros α/β u homodímeros α/α en timocitos y en linfocitos T maduros (125). La proteína β del CD8 se encuentra glicosilada de manera diferente en timocitos y linfocitos T maduros. En el timo, la proteína β del CD8 se encuentra con glicanos O-glicosilados, mientras que en los linfocitos T maduros

está hiposialilada (126). La cadena α del CD8 también es reconocida por la lectina PNA, aun en ausencia de la cadena β (118).

La lectina de Amaranto es una herramienta importante para aislar timocitos medulares (72) y linfocitos humanos CD4+CD45RA+ o en transición (73). El receptor para amaranto es una típica O-glicoproteína sialilada de 70 kDa (127), con una homología <17% con un dominio asociado a la proteína Fas y con el receptor tipo II del factor transformante del crecimiento-ß (127). ALL reconoce GalNAc en la estructura Galß1,3GalNAc; sin embargo, la interacción de ALL con las glicoproteínas muestra que la distribución de los carbohidratos son importantes para la interacción con esta lectina. A diferencia de otras lectinas con especificidad similar como la lectina *Salvia sclarea* (específica para CD43), *Vicia villosa* (específica para linfocitos CD8 supresores) o PNA, la organización tridimensional de los residuos GalNAc juega un papel relevante en los receptores celulares que son reconocidos por la lectina de *A. leucocarpus* (23). Nuestros resultados sugieren que la O-glicosilación, juega un papel relevante en los procesos de maduración y /o activación de los linfocitos porcinos.

# Peanut agglutinin (PNA) and *Amaranthus leucocarpus* (ALL) lectins discriminate between memory and naive porcine lymphocytes

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## Abstract

Lectins are relevant tools to isolate and characterize different cellular sub-populations. In this work we used the lectins Peanut agglutinin (PNA) and *Amaranthus leucocarpus* (ALL), specific for Galß1,3GalNAc, to characterize naïve and memory porcine lymphocytes. Our results showed that both lectins agglutinated preferentially lymphocytes with the CD4+CD8+ phenotype ($p<0.05$), than those lymphocytes CD4+CD8- ($p>0.05$). The phenotypic analysis of the cellular subpopulations recognized by these lectins indicated that PNA+ lymphocytes showed higher rate of the CD29 antigen (PNA+CD29$^{high}$), whereas the ALL+ lymphocytes showed lower rates of this antigen (ALL+CD29$^{low}$). Presence of PNA+CD29$^{high}$ lymphocytes increased after experimental infection with the porcine rubulavirus (RvP), and most of the ALL+CD29$^{low}$ cells became CD29$^{middle}$. The lymphocyte subset recognized by PNA+ from infected pigs proliferated after stimulation with the RvP. In contrast, ALL+ cells were not stimulated by viral recall. Our results suggest that the cellular distribution/organization of the O-glycosydically linked glycans on CD4+CD8+ and CD4+CD8- may correlate with biological functions, and that PNA could be a tool to isolate specifically porcine memory T-cell subsets, whereas ALL could be useful to isolate naive T-lymphocytes.



Key words: swine, CD4+CD8+, peanut agglutinin (PNA), *Amaranthus leucocarpus* lectin, memory, naive, lymphocytes, O-glycosydically linked glycans.

## 1. Introduction

Lectins, which show specificity for O-glycosydically linked glycans (containing the structure Gal ß1,3GalNAcα1,0 Ser/Thr), have been widely used in the fractionation of thymocytes and lymphocyte subpopulations. By selective agglutination with peanut agglutinin (*Arachis hypogaea*), it is possible to purify cortical immature thymocytes (Reisner et al., 1976), and CD8+ T cells activated in vitro (Chervenak and Cohen 1982; Verheijen et al., 1983; Taira and Nariuchi 1988). The lectin from *Amaranthus leucocarpus* (ALL) possesses the capacity to interact with murine medullar thymocytes (Lascurain et al., 1994), and human naive CD4+ lymphocytes (Lascurain et al., 1997). PNA and ALL bind with the highest affinity to the disaccharide Galß1,3GalNAc, a sequence typically found in O-linked glycans (Pereira et al., 1976).

In this study, we examined changes in O-glycosydically linked glycans on T cells during *in vivo* viral infection with the porcine rubulavirus in swine. Adult swine infected with porcine rubulavirus (RvP) showed increased infertility rates in gilts, stillbirths, and mummified fetuses in pregnant sows, and epididymitis and orchitis in boars (Stephano, 1994; Ramirez et al., 1997). Phenotype analysis of peripheral blood mononuclear cells (PBMC) from RvP infected animals revealed increased number of both monocytes (PoM1+) and total T lymphocytes (CD2+) early during infection and both $CD4^{-}CD8^{+}$ and $CD4^{+}CD8^{+}$ cells increased at 4 weeks pi (Hernandez et al., 1998), suggesting that CD4+CD8+ could participate in the

regulation of porcine memory CD8+ T cells (Summerfield et al, 1996; Zuckermann and Husmann 1996; Hernandez et al., 1998).

In contrast to human or murine lymphocytes, it has been found that, in normal adult swine, a substantial proportion (10 to 60%) of their lymphocytes shows the CD4+CD8+ phenotype (Saalmuller et al., 1988). This cellular subset has been identified in peripheral blood, spleen, lymph nodes and tonsils of normal adult swine (Zuckermann and Gaskinz, 1996). Maturation of T cells is assessed by expression of the T cell receptor and the CD4 and CD8; however, CD4+CD8+ lymphocytes exhibit properties of mature antigen experienced cells (Summerfield et al, 1996; Zuckermann and Husmann 1996; Hernandez et al., 1998).

The leukocyte common antigen, CD45, has been considered a potential marker of memory T cells. Resting or naive murine CD4+T cells express a high-molecular weight isoform recognized by anti CD45RB monoclonal antibodies (CD45RA in humans); the reciprocal subsets on activated lymphocytes are identified by the loss of CD45RB monoclonal antibodies staining (Bell *et al.,* 1998). Recent works indicated that the CD29 phenotype could be considered as a potential marker for memory T cell subsets in swine (Zuckermann and Husmann 1996). In this work we examine, with the aid of lectins, changes in the O-glycosylation pattern of porcine T cell surface glycoproteins during *in vivo* activation and explore the possibility that these changes could be used to discriminate among naive, effector, or memory T cells.

## 2. Materials y Methods

### *2 1.Animals*

Crossbred domestic adult pigs (>7 months) were obtained from a commercial farm. Animals are certified to be free of most swine diseases, including porcine rubulavirus. Pigs were experimentally infected with 5 ml rubulavirus ($10^4$ $TCID_{50}$/ml) and placed individually in an isolation facility. The virus was propagated in the pig kidney cell line PK-15 with MEM (supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin). Infected cell cultures were clarified by centrifugation at 3200 rpm 45 min at 4°C and virus infectivity was tittered in cell cultures in 96-wells microculture plates (Falcon Labware, New Yersey, USA). Pigs were killed at the end of the experiment following guidelines for laboratory animals care.

### *2.2. Antibodies*

The following mouse monoclonal antibodies (mAb) were kindly provided by Dr. M. D. Petscovitz (Indiana University, Indianapolis USA): anti-CD4 (74.12.4, IgG2b), anti-CD8 (76.2.11, IgG2a) and anti-macrophage (78.2.11, IgG2a) (Petzcovist 1984). They were revealed with fluorescein isothiocyanate (FITC) labeled anti-mouse IgG2b (Serotec, Oxford, England) and Phycoerythrine (PE) labeled-anti-mouse IgG2a (Zymed Lab Inc., San Francisco CA., USA). RD1-

labeled human β1 integrin-specific mAb 4B4 (CD29) was purchased from Coulter (Coulter Sci.. San Diego CA, USA).

*2.3 Lectins*

*Amaranthus leucocarpus* lectin (ALL) and peanut agglutinin (PNA from Arachis hyogaea) seeds were collected in Tulyehualco (Mexico) and purified on a column containing stroma from human erythrocytes as described previously (Hernández et al., 1998; Ortiz *et al.*, 2000). ALL and PNA lectins were labeled with the N-hydroxysuccinimide ester of biotin from Pierce Chem. Co. (Rockford, IL, USA) at a label/protein ratio of 2:1 (Savage *et al.,* 1984). Biotin-conjugate PNA was used at a concentration of 0.1 mg/$10^6$ cells in the presence or absence of 0.2 M galactose as inhibitor of specific lectin binding. ALL was used at a concentration of 10 µg/$10^6$ cells in the presence or absence of 200 mM GalNAc as inhibitor of specific lectin binding. CyChrome-streptavidin (Pharmigen,Sn Diego CA, USA) was used in conjunction with biotin-conjugate PNA or ALL lectins.

*2.4 Three- and two-color cytofluorometric analysis*

PBMC were stained with anti-CD8, anti-CD4 mAb and biotin-labeled PNA or ALL. After 20-min incubation on ice, cells were washed with PBS (0.1 M, pH 7.2) containing 0.3% of bovine serum albumin; reacted with FITC-labeled rat anti-

mouse IgG2b, PE labeled-rat anti-mouse IgG2a, and CyChrome-streptavidin After a final wash, cells were analysed into CD4+PNA+, CD4+ALL+, CD8+PNA+, CD8+ALL+ and CD4+CD8+PNA+, CD4+CD8+ALL+ subpopulations. For the staining of PNA+CD29+ and ALL+CD29+ lymphocytes, PBMC were stained with Biotin-conjugate PNA or Biotin-conjugate ALL. After washing, CyChrome-streptavidin and RD1-labelled anti-human CD29 mAb were added. After final washing, cells were analyzed through flow cytometry with a Cell Sorter FACS Excalibur (Becton & Dickinson, Mountain View, CA, USA).

*2.5 Electronically-sorting of PNA+ and ALL+ lymphocytes*

PNA+ and ALL+ lymphocytes were isolated by Two-color FACS with a Cell Sorter FACS Excalibur. Eppendorf tubes containing suspensions of glass-adherent cell-depleted PBMC ($10^7$/tube) in sterile flow PBS were reacted with anti-CD8 mAb and biotin-conjugate PNA or biotin-conjugate ALL. After 20 min incubation on ice, cells were washed with PBS and reacted with FITC-labeled rat anti-mouse IgG2b and CyChrome-streptavidin. After a final wash, cells were suspended at $10^7$/ml in PBS and sorted into CD8+PNA+, CD8+PNA-, CD8+ALL+ and CD8+ALL-. For all experiments shown, cytofluorometric analysis of sorted cells showed >94% of the sorted phenotype.

*2.6 Isolation of Antigen Presenting Cells*

A single cell suspension of PBMC was stained with 100µl of a mouse anti-pig monocyte-monoclonal antibody at 4°C during 15 min. Cells were washed with 0.1 M PBS, pH 7, and 0.3% BSA and incubated with sheep anti-mouse immunoglobulin-coated magnetic particles using a magnetic sorter (MACS. Miltenyi Biotec Inc., Sunnyvale, CA, USA). The purified cells were >96% as assessed by FACScan.

*2.7 Lymphoproliferation assays*

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples by Ficoll-Hypaque (Pharmacia, Sweden) gradient centrifugation. Lymphocytes subpopulations were isolated from PBMC from infected and naive pigs. Lymphocytes were cultured at 2.5 x $10^5$/well in sterile 96-wells round bottom plates in 200 µl RPMI culture medium (HEPES-buffered RPMI-1640 (Sigma Chemicals, St Louis, MO., USA).supplemented with 2 x $10^{-5}$ m 2-mercaptoethanol, 2mM sodium pyruvate, 2mM L-glutamine, 1 µg/ml of Gentamycin, and 10% of fetal calf serum). Lymphocytes were stimulated with viral antigen (10 µg/ml) for 5 days in a humidified incubator with 5% $CO_2$ at 37° in the presence of 0.25 x $10^5$ autologous antigen presenting cells (APC). Viral antigen consisted of supernatants of infected pig kidney (PK) cells and concentrated by ultracentrifugation at 100,000

*g*, 4 h. at 4°C. filtered through 0 45-μm membranes, aliquoted and stored at -70°C until use. All experiments were performed in triplicate. Proliferation was assayed by the addition of 50μl/weell of culture medium contained 1μCi of 3Hthymidine (New England Nuclear, specific activity 52 Ci/mmol) during the last 16-18 h of culture. Cells were harvested onto fiberglass filter paper with a cell harvester, and the incorporated radioactivity was measured with a Beckman LS6000SE SE-counter (Beckman. Fullerton CA.) and used as a mean of lymphocyte proliferation. Results are expressed as Stimulation Index (S.I.)

## 3. Results

### *3.1 PNA+ and ALL+ lymphocyte subset characterization*

To determine the expression of O-glycosylated structures on the subsets of peripheral lymphocytes, triple-marker immunostaining was performed to analyze recognition of PNA or ALL lectins independently. To determine the specific staining of the lectins their recognition was inhibited with specific sugars, lactose for PNA and GalNAc for ALL. Figure 1 shows the proportion of lymphocyte subsets identified in the porcine peripheral blood cells corresponding to CD4-CD8- (34%), CD4+CD8- (20%); CD4-CD8+ (39%), and CD4+CD8+ (7%). These cellular populations were differentially recognized by ALL and PNA lectins (Figure 1). Our results indicate that PNA recognized the four cellular populations of lymphocytes: 11±% of CD4+CD8- lymphocytes, 14±5% of CD4-CD8+, 32±14% of CD4-CD8-, and 44±6% of CD4+CD8+ T lymphocytes. ALL also identified the four cellular subsets: 11±4% of CD4+CD8-, 27±8% of CD4-CD8+, 27±5% CD4-CD8-, and 42±6% of CD4+CD8+ lymphocytes. These results indicate that PNA recognized preferentially lymphocytes CD4+CD8+ ($p<0.05$) and ALL recognized preferentially lymphocytes CD4-CD8+ and CD4+CD8+ ($p<0.05$) (Table 1).

*3.2. PNA+ lymphocytes contained cells with a memory phenotype, while ALL+ lymphocytes are naive cells*

To determine whether the lymphocytes recognized by the lectins belong to memory/effector cells, we used antibody anti-CD29. Results indicate that PNA+ CD4+CD8-, CD4-CD8+, and CD4+CD8+ lymphocytes showed to be CD29+ ($PNA+CD29^{High}$, figures 2 and 3). Lymphocytes PNA+CD4-CD8-, as well as the ALL+ lymphocytes, were CD29 with low levels of recognition ($ALL+CD29^{Low}$). These results indicate that PNA+ lymphocytes showed the memory phenotype, and ALL+ lymphocytes corresponded to naïve cellular subsets (Figure 2).

*3.3 PNA+ memory-lymphocytes increased after infection*

After immunization with 5 ml of $10^4$ $TCID_{50}$/ml of RvP, the proportion of PNA+ CD4+CD8+ lymphocytes showed a two-fold increase at week 8 post-inoculation (pi) as compared to non-immunized organisms, this increase was also associated with the increased capacity to respond to virus recall in *in vitro* activation, as a secondary immune response specific to the virus. In the presence of PNA or ALL lectins, our results indicate that the proportion of CD4+CD8+ $PNA+CD29^{High}$ also increased significantly in the immunized animals ($p<0.01$) as compared to non-infected animals. After virus recall the ALL+ cells showed a light increase in the expression of CD29 which appeared as $CD29^{middle}$ (Figure 2).

*3.4 PNA+ cells are responsive to recall viral antigen*

To identify the possible participation of PNA+ lymphocytes in memory/effector activity, we analyzed the capacity of electronically sorted PNA+ and ALL+ peripheral blood lymphocytes from an RVP-infected pig to mediate a secondary response to viral antigen. Cytofluorometric analysis of sorted $CD8^{low}PNA+$, $CD8^{low}PNA-$, $CD8^{low}ALL+$, and $CD8^{low}ALL-$ lymphocytes showed their purity to be greater than 93% in all experiments. Our results show that both $CD8^{low}PNA+$ and $CD8^{low}ALL-$ cell populations, when isolated from RvP-infected pigs, were able to respond to stimulation with equal doses of recall viral antigen (Stimulation index (SI)>9). However, $CD8^{low}PNA-$ and $CD8^{low}ALL+$ lymphocytes, when isolated form RvP-infected pigs, were unable to respond to recall (SI=3). The $CD8^{low}PNA+$ and $CD8^{low}ALL-$ cells when isolated from naive pigs were unable to respond in the presence of the virus. Since both $CD8^{low}PNA+$ and $CD8^{low}ALL-$ lymphocytes were able to respond to stimulation with recall viral antigen, these results indicate that memory/effector T cells are present in both lymphocyte populations, and that $CD8^{low}PNA-$ and $CD8^{low}ALL+$ lymphocytes contained naive cells (Figure 3).

## 4. Discussion

The glycosylation pattern on T cell surface glycoproteins changes as cells in the thymus mature and migrate to the periphery (Gillespie et al., 1993). In swine, as in other animal species, maturation of T cells is assessed by expression of the T cell receptor and the CD4+ and CD8+ co-receptor molecules; furthermore, CD4+CD8+ lymphocytes, in swine, exhibit properties of mature antigen experienced cells (Summerfield et al, 1996; Zuckermann and Husmann 1996; Hernandez et al., 1998). For this reason it would be useful to identify unique oligosaccharides that characterize T cell subsets, especially if these structures can be used to distinguish memory and effector T cells from naive T cells. In this study, we showed that PNA cells become $CD29^{High}$; whereas naïve $CD29^{low}$ lymphocytes remain positively recognized by the lectin from *Amaranthus leucocarpus*, indicating altered expression or three-dimension organization of O-glycans as occurred during activation of porcine T-cells.

Cells from RvP-infected pigs were stained with PNA or ALL to determine the expression pattern of the lectin's specific O-glycosydic-ligand. The virus-induced activation, produced an expansion of CD4+CD8+ cells. Most CD4+CD8+ populations stained PNA+. The increase in PNA binding correlated with increased of CD4+CD8+ cells, which were also $CD29^{High}$. The proportion of ALL+ $CD29^{low}$, which seems to correspond to naïve cell subsets, remained unaltered after RvP infection. Porcine CD29 is the ß1 subunit of the integrin that functions as a receptor for cell adhesion molecules of the extracellular matrix, it is involved in

rejection of pig-to-human tissue xenografts, and is involved in homing and differentiation of hematopoietic progenitor cells (Jimenez-Marin et al . 2000). These data show that the increase in PNA binding T cells is a general property of memory T cell activation during viral infection and also confirming that porcine CD4+CD8+ lymphocytes have memory functions (Summerfield et al, 1996; Zuckermann and Husmann, 1996; Hernández et al., 1998).

The memory phenotype on PNA+ lymphocytes provide support for the hypothesis that these cells might include memory/effector T cells, and the naive phenotype on ALL+ lymphocytes might contain cells unable to respond in a viral-antigen assay. To prove this hypothesis, the capacity of electronically sorted PNA+ and ALL+ peripheral blood lymphocytes to mediate a secondary response to viral antigen was analyzed. Since PNA and ALL lectins recognize mainly CD4+CD8+ lymphocytes, electronically cells recognized by either lectin or by the mAb against CD8 to purify $CD8^{low}PNA+$ and $CD8^{low}ALL+$ cells. Cytofluorometric analysis of sorted $CD8^{low}PNA+$, $CD8^{low}PNA-$, $CD8^{low}ALL+$ and $CD8^{low}ALL-$ lymphocytes showed that both $CD8^{low}PNA+$ and $CD8^{low}ALL-$ cell populations from RvP-infected pigs were able to respond to stimulation with equal doses of recall viral antigen, whereas $CD8^{low}PNA-$ and $CD8^{low}ALL+$ lymphocytes form RvP-infected pigs were unable to respond to recall. As demonstrated in murine CD8+ T cells, (Harrington et al., 2000) our results confirmed that porcine $CD8^{low}PNA+$ and $CD8^{low}ALL-$ lymphocytes correspond to memory/effector T cells populations.

PNA binds to Gal ß1,3GalNAc; however, when sialic acid is present, the PNA binding site is masked. It has been suggested that activation of

memory/naïve CD8+ cell subsets induces a decrease in the expression or activity of sialyltransferase enzymes in T cells, which may result in the expression of desialylated surface glycoproteins able to bind PNA (Galvan et al., 1998). PNA binds to CD43, CD45 (De Maio et al., 1986; Wu et al., 1996); furthermore, it has been shown that during murine T cell activation, O-linked sugars on the CD8 chain interact with PNA (Casabo et al., 1994). Since the level of interaction of PNA with CD4+CD8+ $CD29^{High}$ T cells increased significantly in infected animals, it is possible that PNA staining might identify cell surface molecules able to distinguish memory from naive cells (Harrington et al., 2000). Our findings show the presence of a double phenotype CD4+CD8+ cellular subset that corresponds to naïve or probably quiescent cell populations, since they showed to be ALL+ and $CD29^{low}$.

The lectin from *A. leucocarpus* is specific for GalNAc in the Galß1,3GalNAc sequence (Zenteno et al., 1992). The lymphocyte O-linked glycans, reactive to ALL, are present in murine medullar lymphocytes (Lascurain *et al.,* 1994) and human T-cells with the CD4+CD45Ra+CD27+ phenotype (Lascurain et al., 1997), indicating that the lectin receptor is present specifically in naive or quiescent cell subpopulations, which could be destined for export to the peripheral lymphoid tissues (Lascurain *et al.,* 1997). The murine lymphocyte receptor for ALL is a glycoprotein of 70 kDa and it is a typical sialylated O-glycosylprotein; this receptor showed low (<17%) homology with Fas-associated death domain protein, and transforming growth factor-ß type II receptor (Porras et al., 2000). The interaction of ALL with glycoproteins showed that the patterns of Galß1,3GalNAc distribution are relevant for the interaction with this lectin (Zenteno et al., 1992). In contrast to

other lectins with similar sugar specificity, such as the lectins from *Salvia sclarea* (specific for CD43) (Piller et al., 1988), *Vicia villosa* (specific for CD8+ contrasuppresor T cells) (Fortune and Lehner,1988) and PNA, adjacent or clustered Galß1,3GalNAc residues on the cellular receptors are not recognized by ALL (Oxenius et al.,1995; Hernández et al., 1999).

The O-glycosylation changes documented in this study could be important for trafficking and localization of memory/effector cells to sites of antigen presentation. Alterations in cell surface glycosylation may also affect T cell recognition of APCs, or interactions with accessory molecules. For example, CD23 on B cells has been shown to bind to the Gal ß1,3GalNAc sequence (Kijimoto-Ochiai et al., 1995), but this sequence can be masked by the addition of sialic acid, resulting in the inhibition of CD23 binding. The dynamic modulation of T cell surface glycosylation indicates that these changes affect T cell function, in addition to allowing the phenotypic discrimination between naive and effector/memory T cells (Harrington et al., 2000; Lascurain et al., 1997; Porras et al., 2000).

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## References

Bell, E.B., Sparshott S.M., and Bunce, C., CD4+ T-cell memory, CD45R subsets and the persistence of antigen-a unifying concept. Immunology Today 1998, 19.61-64.

Casabo, L. G., Mamalaki, C., Kioussis, D., and Zamoyska, R. 1994. T cell activation results in physical modification of the mouse CD8 chain. J. Immunol. 152, 397-401.

Chervenak, R., and Cohen J. J. 1982 Peanut lectin binding as a marker for activated T-lineage lymphocytes. Thymus 4, 61-67.

De Maio, A., Lis, H., Gershoni, J.M., and Sharon, N. 1986.Identification of glycoproteins that are receptors for peanut agglutinin on immature (cortical) mouse thymocytes. FEBS Letters. 194, 28-32.

Fortune, F., and Lehner, T. 1988. Phenotypic expression of *Vicia villosa* binding T cell subsets, as markers of contrasuppressor cells in systemic lupus erythematosus. Clin. Exp. Immunol. 74, 100-104.

Galvan, M., Murali-Krishna, K., Lua, L., Baum, L., and Ahamed, R. 1998. Alterations in cell surface carbohydrates on T cells from virally infected mice

can distinguish effector-memory CD8+ T cells from naive cells. J. Immunol. 161, 641-648.

Gillespie, W., Paulson, J.K., Kelm, S., Pang, M., and Baum, L.G. 1993. Regulation of alpha 2,3-sialyltransferase expression correlates with convertion of peanut agglutinin (PNA)+ to PNA- phenotype in developing thymocytes. J. Biol. Chem. 268, 3081-3089.

Harrington, L.E., Galvan, M., Baum, L. G., Altman, J. D., and Ahmed, R. 2000 Differentiating between memory and effector CD8 T cells by altered expression of cell surface O-glycans. J. Exp. Med.191, 1241-1246.

Hernández, M. Bacilio, F. Porras, S. Juarez, H. Debray, E. Zenteno, and Ortíz B. 1999. A comparative study on the purification of the *Amaranthus leucocarpus* syn. *hypocondriacus* lectin. Prep. Biochem. Biotech. 29, 219-234.

Hernández J., Reyes-Leyva J., Zenteno R., Ramirez H., and Zenteno E. 1998 Immunity to porcine rubulavirus infection in adult pigs. Vet Immunol Immunopathol 64, 367-381.

Kijimoto-Ochiai, S., and Uede, T. 1995. CD23 molecule acts as galactose-binding lectin in the cell aggregation of EBV-transformed human B-cell lines. Glycobiology 5, 443-451.

Jimenez-Marin, A., Garrido, J., de Andres-Cara, D. F., Morera, L., Barbancho, M.J., and Llanes, D.. 2000. Molecular cloning and characterization of the pig homologue to human CD29, the integrin beta1 subunit. Transplantation 70, 649-55.

Lascurain, R., Chavez, R., Gorocica, P., Perez, A., Montano, L. F., and Zenteno, E. 1994. Recognition of a CD4+ mouse medullary thymocyte subpopulation by *Amaranthus leucocarpus* lectin. Immunology 83, 410-413.

Lascurain, R., Porras, F., Baez R., Chavez, R., Martinez-Cairo S., Alvarez G., and Zenteno, E. 1997. *Amaranthus leucocarpus* lectin recognizes human naive T cell subpopulations. Immunol. Invest. 26, 579-587.

Ober, B., Summerfield, A., Mattlinger, C., Wiesmuller, K., Jung, G., Pfaff, E., Saalmuller, A., and Rziha, H. 1998. Vaccine-induced, pseudorabies virus-specific, extrathymic CD4+CD8+ memory t-helper cells in swine. J. Virol. 72, 4866-4871.

Ortiz, B., Bacilio, M., Gorocica P., Montaño, L. F., Garfias, Y, Zenteno, E. 2000. The hydrophobic character of peanut (*Arachis hypogaea)* isoagglutinins Journal of Agricultural and Food Chemistry (In Press).

Oxenius, A., Bachmann, M. F., Ashton-Rickardt, P.G., Tonegawa, S., Zinkernagel, R. M., and Hengartner, H. 1995. Presentation of endogenous viral proteins in association with major histocompatibility complex class II: On the role of intracellular compartmentalization, invariant chain and the TAP transporter system. Eur. J. Immunol. 25, 3402.-3412.

Piller, F., Piller, V., Fox, R. I., and Fukuda, M. 1988. Human T- lymphocyte activation is associated with changes in O- glycan biosynthesis. J. Biol. Chem. 263, 15146-15150.

Pereira, M. E., Kabat, E. A., Lotan, R., and N. Sharon, N. 1976. Immunochemical studies on the specificity of the peanut (*Arachis hypogaea*) agglutinin. Carbohydr. Res. 51, 107-110.

Pescovitz, M.D., Lunney, J.K., and Sachs, D.H. 1984. Preparation and characterization of monoclonal antibodies reactive with porcine PBL. J. Immunol. 133, 368-373.

Porras, F., Lascurain, R., Chávez, R, Ortiz, B., Hernández, P., Debray, H., and Zenteno, E. 2000. Isolation of the receptor for *Amaranthus leucocarpus* lectin from murine naïve thymocytes Glycobiology 10, 459-465.

Ramírez-Mendoza, H., Hernández-Jáuregui, P., Reyes-Leyva, J., Zenteno, E., Moreno-López, J., and Kennedy, S 1997. Lesions in the reproductive tract of boars experimentally infected with porcine rubulavirus. J. Comp. Path. 117, 237-252.

Reisner, Y., Linker-Israeli, M., and Sharon, N. 1976. Separation of mouse thymocytes into two subpopulations by the use of peanut agglutinin. Cell. Immunol. 25, 129-134.

Saalmueller, A., Redheads, M. J., Buhring, H. J., Jonjic, S., and Koszinowski, U. H. 1987 Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. Eur. J. Immunol. 17, 1297-1302.

Savage, D., Mattson, G., Desai, S., Nielander, G., Morgensen, S., and Conklin, E. eds. 1992. Avidin-biotin chemistry: A handbook. Ed. Pierce Chemical Company, Rockford, IL, USA.

Summerfield, A., Rziha, H., and Saalmueller, A. 1996. Functional characterization of porcine CD4+CD8+ extrathymic T lymphocytes. Cellular Immunology 168, 291-299.

Stephano, H.A. 1994. Blue eye disease, p. 237-241. In: W.L. Mengelin (ed.) Diseases of swine. Sixth ed. Ames, Iowa State University Press, US.

Taira, S., and H. Nariuchi. 1988. Possible role of neuraminidase in activated T cells in the recognition of allogeneic Ia. J. Immunol. 141, 440-442.

Verheijen, F. W., Janse, H. C., van Deggelen, O. P., Bakker, H. D., Loonen, M. C.B., Durand, P., and Galjaard, H. 1983. Two genetically different MU-NANA* neuraminidases in human leukocytes. Biochem. Biophys. Res Commun 117, 470-476.

Wu, W., Harley, P. H., Punt, J. A., Sharrow, S. O., and Kearse K. P. 1996. Identification of CD8 as a peanut agglutinin (PNA) receptor molecule on immature thymocytes. J. Exp. Med. 184, 759-767.

Zenteno, E., Lascurain, R., Montaño, L. F., Vazquez, L. Debray, H. and Montreuil J. 1992. Specificity of *Amaranthus leucocarpus* lectin. Glycoconjugate J. 9, 204-208.

Zuckermann, F. and Gaskins, H.R. 1996. Distribution of porcine CD4/CD8 double-positive T lymphocytes in mucosa-associate lymphoid tissues. Immunology 87, 493-504.

Zuckermann, F., and Husmann, R. 1996. Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. Immunology 87, 500-508.

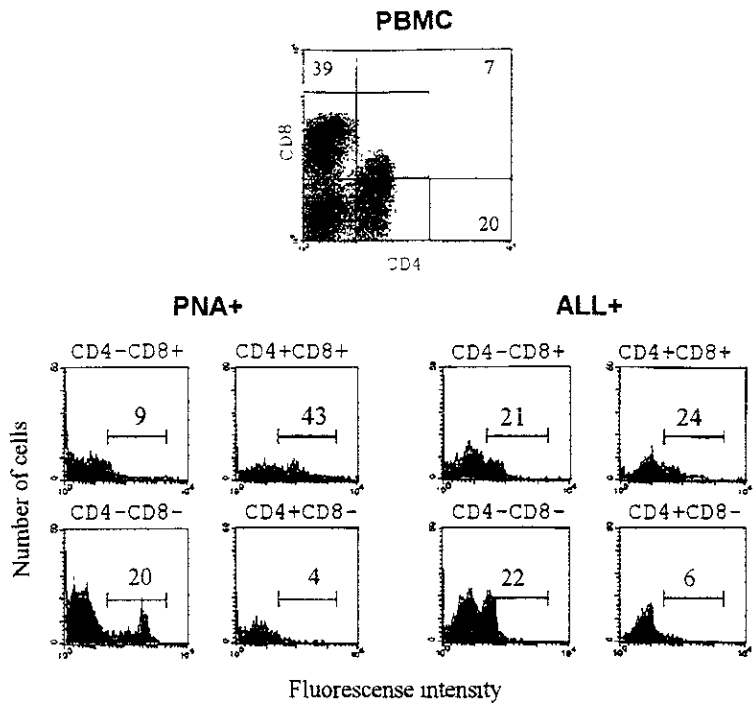


Figure 1. Expression of O-glycosylated structures on the subsets of peripheral lymphocytes. Representative FACS analysis of CD4 and CD8 expression on PBMC isolated form a naive pig (A), which describes the binding of PNA and ALL lectins by recognition O-glycosylated structures on subsets of peripheral lymphocytes.

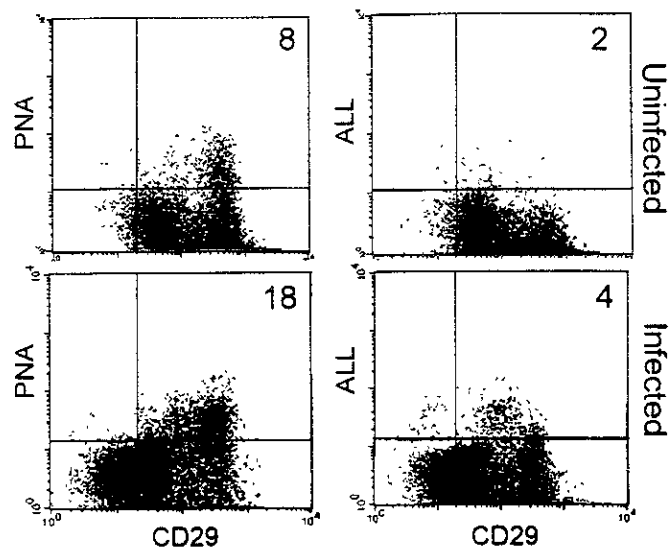


Figure 2. Two-color cytofluorometric analysis of PBMC. Porcine PBMC from a naive pig (upper) and from a pig after 2 months of infection pig (bottom) were stained with biotin-conjugate PNA or ALL lectins and mAb anti-CD29. Numbers represent the percentages of positive cells within each quadrant.

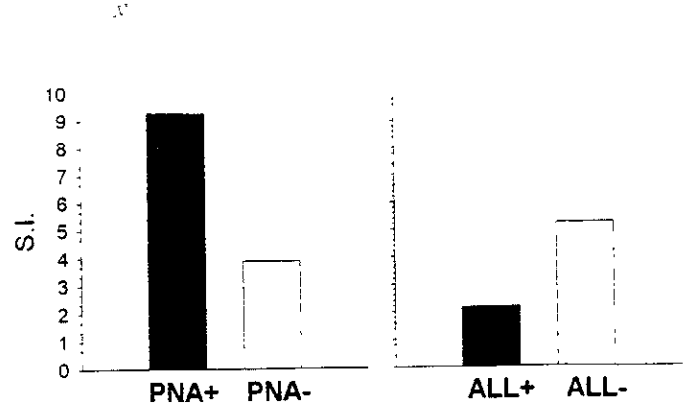


Figure 3. PNA+ lymphocytes respond to a recall viral antigen. PNA+, PNA-, ALL+, and ALL- lymphocytes from infected pigs were electronically sorted and cultured with autologous antigen presenting cells in presence of viral antigen. Proliferative response of flow cytometric-separated T subpopulations was quantified by $^{3}H$ thymidine incorporation and expressed as the Stimulation Index (SI). Our results show that both $CD8^{low}PNA+$ and $CD8^{low}ALL-$ cell populations, when isolated from RvP-infected pigs, were able to respond to stimulation with equal doses of recall viral antigen (SI >9).

Table 1. Analysis of the expression of O-glycosylated structures on porcine T-lymphocytes

| Subset | PNA | ALL |
|---|---|---|
| | Recognized cells (%) | |
| CD4+CD8- | 11±7 | 11±4 |
| CD4+CD8+ | 44±6* | 42±6** |
| CD4-CD8+ | 14±5 | 27 ±8 |
| CD4-CD8- | 32±14 | 27±5 |

PNA recognized preferentially lymphocytes CD4+CD8+ (*p<0 05) and ALL recognized lymphocytes CD4-CD8+, CD4-CD8- and CD4+CD8+ (**p<0.05).

## CONCLUSIONES

Este trabajo describe los primeros resultados que ayudan a entender el desarrollo de la respuesta inmune al rubulavirus porcino y propone nuevas herramientas que permitirán conocer más a fondo los mecanismos involucrados en la memoria inmunológica a este virus y en general a la memoria inmune del cerdo.

- Este trabajo demostró que la infección genera anticuerpos específicos contra el virus a partir de la 1ª semana de infección y se mantienen hasta la 7ª semana que duró el experimento. Los resultados mostraron que la proteína HN es la proteína más inmunogénica del virus, seguida por las proteínas M y NP. De acuerdo a los resultados, la proteína F es la menos inmunogénica. Por lo que se propone que la HN sea el candidato para desarrollar técnicas de diagnóstico y vacunas para el control de la enfermedad.
- Durante las primeras semanas de infección, la respuesta proliferativa a las lectinas disminuyó significativamente en los cerdos infectados ($p<0.05$), lo que implica una disminución importante de la respuesta inmune de tipo celular. Después de la 3ª semana de infección no se observaron modificaciones ($p>0.05$).
- Se demostró que las CMN de los cerdos infectados proliferan *in vitro* cuando se cultivan en presencia del virus homólogo ($p<0.05$). La respuesta específica se presentó a partir de 2ª semana de infección y se mantuvo durante las siete semanas que duró el experimento.
- El fenotipo de las CMN que proliferaron en presencia del virus mostró un incremento de linfocitos CD4+CD8- y CD4+CD8+, siendo mayor el incremento de las células CD4+CD8+ ($p<0.05$). Las CMN estimuladas con PHA sólo incrementaron el fenotipo CD4+CD8- y las CMN de los cerdos testigos permanecieron sin cambios ($p>0.05$).

- Durante la 1ª semana de infección se incrementaron los porcentajes de linfocitos CD4-CD8+, y durante la 3ª semana disminuyeron los CD4+CD8- ($p<0.05$).
- Este trabajo es el primero en describir las modificaciones *in vivo* de los linfocitos CD4+CD8+ después de una infección experimental. Los resultados revelaron que esta subpoblación incrementa significativamente ($p<0.05$).
- Los resultados apoyan la hipótesis de la participación de los linfocitos CD4+CD8+n la regulación de la respuesta inmune.
- Los linfocitos CD4+CD8- participan en las primeras semanas de infección. Después de analizar el fenotipo de las células estimuladas con virus, los resultados revelaron que más del 50% de los linfocitos CD4+CD8- expresaron la molécula CD8 y se convirtieron en linfocitos CD4+CD8+.
- Los linfocitos CD4+CD8+ participaron en la respuesta de memoria al virus. Estas células no modificaron el fenotipo después del estímulo con virus, PHA o en las células control.
- Los linfocitos CD4+CD8+ presentaron un perfil de citocinas similar a una célula de memoria (<IL-2, >IL-10), mientras que en los linfocitos CD4+CD8- el perfil fue de una célula virgen (>IL-2, <IL10).
- Estos resultados confirmaron que los linfocitos CD4+CD8+ participan en la memoria inmune al virus y confirman la hipótesis original que planteaba que esta subpoblación era una importante estirpe celular en la memoria inmunológica del cerdo.
- La lectina PNA reconoció las diferentes subpoblaciones de linfocitos de sangre periférica (CD4+CD8-, CD4-CD8+, CD4+CD8+ y CD4-CD8-); sin embargo, su reconocimiento fue significativamente mayor por los linfocitos CD4+CD8+ ($p<0.05$).
- La lectina ALL presentó un comportamiento similar; sin embargo, ALL además reconoció linfocitos CD4-CD8+.

- El análisis del fenotipo de las células PNA y ALL reveló que la lectina PNA reconoció linfocitos de memoria y ALL reconoció células vírgenes o en transición.
- El análisis de las células PNA+CD29+ y ALL+CD29- después de la infección indicó que la infección con el rubulavirus incrementó los porcentajes de las primeras ($p<0.05$) y que las poblaciones celulares reconocidas por ALL permanecieron constantes ($p>0.05$).
- Después de aislar linfocitos PNA+ y ALL+ y evaluar su capacidad para responder en una respuesta secundaría *in vitro* en presencia del virus, únicamente los linfocitos PNA+ tienen la capacidad de responder en presencia del virus ($p<0.05$).
- Estos resultados confirman que la lectina PNA se puede usar para el estudio y caracterización de los linfocitos de memoria del cerdo, mientras que la lectina ALL para los linfocitos vírgenes.

## PERSPECTIVAS

Este trabajo presenta resultados que han permitido evaluar la respuesta inmune al rubulavirus porcino y la respuesta inmune del cerdo en general. Sin embargo, son muchas las incógnitas que este trabajo ha despertado:

a) Estudios recientes indican la existencia de nuevos aislamientos del rubulavirus, lo que supone la existencia de algunas variantes del virus. De acuerdo con nuestros resultados, una de las proteínas que deberá caracterizarse con mayor detalle es la HN.

b) El incremento en los porcentajes de linfocitos CD4-CD8+ que se asocia con la expresión de citocinas tipo Th1, deja abierta la pregunta sobre la importancia de la respuesta citotóxica en el control de la enfermedad.

c) La disminución en los porcentajes de linfocitos CD4+CD8- durante el curso de la respuesta inmune, se aclarará con la identificación de esta población en cortes histológicos de los sitios de infección.

d) Se logró identificar la relevancia de los linfocitos CD4+CD8+ en la respuesta inmune al rubulavirus, sin embargo, aún es necesario determinar si como las células de memoria del hombre, estos linfocitos se encuentran protegidos contra la apoptosis, y si esta población es dependiente de antígeno.

e) La capacidad de la lectina PNA para reconocer células de memoria permite proponer que podría utilizarse como un marcador de la respuesta celular para caracterizar de una manera más completa la respuesta inmune del cerdo a los diferentes antígenos.

f) La capacidad de las lectinas PNA y ALL para reconocer linfocitos CD4+CD8+ abre la posibilidad de contar con un marcador que permita el aislamiento y caracterización de esta subpoblación.

## REFERENCIAS


1. Stephano H.A., Ramírez T.C., Gay G.M., y Maqueda J.J. (1981) Estudios de un brote de encefalitis en lechones por un virus hemaglutinante. Memorias del XVII del Congreso de la Asociación de Médicos Veterinarios Especialistas en Cerdos.
2. Stephano H.A., Gay G.H., and Ramires T.C. (1998) Encephalomyelitis, reproductive failure and corneal opacity (Blue eye) in pigs, associated with a paramixovirus infection. Vet. Rec. 122: 6-10.
3. Stephano H.A. 1994. Blue Eye diseases. In: Leman, A.D., Straw, B.F., Mengeling W.L., Allaire, A.D., Taylor, D.J., (Edltors.) Swine Diseases, Iowa State University press, pp. 237-241.
4. Rima B., Alexander D.J., Billeter M.A., Collins P.L., Kingsbury D.W., Lipkind M.A., Nagai Y., Örvell C., Pringle C.R., and ter Mullen V. (1995) Family *Paramyxoviridae*, p. 265-274. In: F.A. Murphy; C.M. Fauquet; D.H.L. Bishop; S.A. Ghabrial; A.W. Jarvis; G.P. Matelli; M.A. Mayo & M.D. Summers (ed.) Virus taxonomy. Classification and nomenclature of viruses. Viena Nueva York: Springer-Verlag.
5. Moreno-López J., Correa-Girón P., Martínez A., and Ericsson A. (1986) Characterization of a paramixovirus isolated from brain of a piglet in México. Arch. Virol. 91: 221-231.
6. Ramírez H., y cols. En preparación.
7. Hjertner B., Linne T., and Moreno-Lopez J. (1997) Establishment and characterisation of a porcine rubulavirus (LPMV) persistent infection in porcine kidney cells. Acta. Vet. Scand. 38: 213-24.
8. Sundqvist A., Berg M., Hernández-Jáuregui P., Linné T., and Moreno-López J. (1991) The haemaglutinin-neuraminidase gene of the porcine paramyxivirus LPMV: comparison with other paramyxovirus revealed the closest to simian virus 5 and mumps virus. Arch. Virol. 122: 331-340.

9 Garfias Y. Tesis de Licenciatura (1998). Facultad de Medicina, UNAM.

10. Hernández J., Ramírez H., Carreón R., Mercado C., Lascuraín R., Hernández-Jáuregui P., Reyes-Leyva J., and Zenteno E. (1994) Several isolates of the Porcine Paramixovirus can be sorted into two groups with diferents behavior. 3rd International Congress of Veterinary Virology, Interlaken, Suiza.

11. Santos G. Tesis de Maestría (2000). Benemérita Universidad Autónoma de Puebla.

12. Ramírez-Mendoza H., Hernández-Jauregui P., Reyes-Leyva J., Zenteno E., Moreno-Lopez J., and Kennedy S. (1997) Lesions in the reproductive tract of boars experimentally infected with the porcine rubulavirus. J. Comp. Path. 117: 237-252.

13. Sundqvist A., Berg M., Hernández-Jáuregui P., Linné T., and Moreno-López J (1990) The structural proteins of a porcine paramyxovirus (LPMV). J. Gen. Virol. 71: 609-613.

14. Lamb,R.A. and Kolakofsky D. (1996) *Paramyxoviridae:* The viruses and their replication, p. 1177-1204. In: B.N. Fields; D.M. Knipe; P.M. Howley et al. (ed.) *Fields Virology* Third ed. Lippincott-Raven Publishers.

15. Reyes-leyva J., Hernández -Jáuregui P., Montaño L.F., and Zenteno E. (1993) The porcine paramixovirus LPM specifically recognize sialyl ($\alpha$ 2,3) lactosa - containing structures. Arch. Virol. 133: 195-200.

16. Reyes-Leyva J., Espinosa B., Hernandez J., Zenteno R., Vallejo V., Hernandez-Jauregui P., and Zenteno E. (1997) NeuAc$\alpha$2,3Gal-Glycoconjugate expression determines cell susceptibility to the porcine rubulavirus LPMV. Comp. Biochem. Physiol. 118B: 327-332.

17. Vallejo V., Reyes-Leyva J., Hernández J., Ramírez H., Delannoy P., and Zenteno E. (2000) Differential expression of sialic on porcine organs during the maturation process. Comp. Biochem Physiol. B. Biochem. Mol. Biol. 126: 415-24.

18. Reyes-Leyva J. (1998) Mecanismos de respuesta al Paramixovirus porcino LPM. Tesis Doctoral, Ciencias Biomédicas, UNAM. México, D.F.

19. Rosales F., Martínez A. y Correa P. (1988). Análisis retrospectivo (1972-1986) de la presencia de anticuerpos inhibidores de la hemaglutinación contra el paramixovirus porcino LPM, al perecer asociados al síndrome del Ojo Azul. Tec. Pec. Mex. 26: 304-311.
20. Carreón R., Fuentes M., Stephano H.A., y Ramírez M.H. (1990) Estudios preeliminares del paramixovirus del ojo azul en la República Mexicana, p. 78-82. In: Doporto JM, Pérez (ed.) Memorias del curso de actualización en enfermedades del cerdo. Ed. UNAM-AMVEC. México.
21. Fuentes R.M., Carreón N.R., Ramírez M.H., Trujillo M.E., y Fraire B.I. (1992) Estudio piloto de la frecuencia de anticuerpos contra el paramixovirus del ojo azul en cerdos de la República Mexicana. Vet. Mex. 23: 37-42.
22. Ramírez-Herrera M.A., Mendoza-Magaña M., and Dueñas S.H. (1997) Experimental infection of swine and cat central nervous systems by the pig paramyxovirus of the blue eye disease. J. Vet. Med. B44: 461-476.
23. Allan G.M., McNeilly F., Walker I., Linné T., Moreno-López J., Hernandez P., Kennedy S., Carroll B.P., Herron H., Foster J.C., and Adair B. (1996) A sequential study of experimental paramixovirus (LPMV) infection of pigs: immunostaining of cryostat sections and virus isolation. J. Vet. Diagn. Invest. 8: 405-413.
24. Wiman A.C., Hjertner B., Linne T., Herron B., Allan G., McNeilly F., Adair B., Moreno-Lopez J., and Berg M. P. (1998) Porcine rubulavirus LPMV RNA persists in the central nervous system of pigs after recovery from acute infection. J. Neurovirol. 4: 545-52.
25. Campos H.R. Y Carvajal S.M. (1989) Trastornos reproductivos en los sementales de una granja porcina de ciclo completo ante un brote de ojo azul. Memorias del XXVI Congreso de la Asociación de Medicos Veterinarios Especialistas en Cerdos. p. 62-64.
26. Hernández-Jáuregui P., Sundqvist A . Fuentes M., Díaz A., Reyes-Leyva J., Hernández E y Moreno-López J. (1992). Correlación entre las pruebas de virus neutralización, inhibición de la hemoaglutinación y ELISA en sueros vacunales

y de brote para anticuerpos contra el Paramixovirus del Síndrome del Ojo Azul en cerdos. Vet. Mex. 23: 217-222.

27. Ramírez-Mendoza H., Carreon N.R., Mercado G.C., and Rodríguez T.J. (1996) Hemaglutinación e Inhibición de la hemoaglutinación del paramixovirus porcino a través de la modificación de algunas variables que participan en la prueba. Vet. Mex. 27: 257-260.
28. McNeilly F., Walker I., Allan G.M., Foster J.C., Linné T., Merza M., Hernandez P., Kennedy S., and Adair B., (1997) A comparative study on the use of virus and antibody detection techniques for the diagnosis of La Piedad Michoacan paramyxovirus (LPMV) infection in pigs. J. Vet. Diagn. Invest. 9: 3-9.
29. Doherty P.C., Topham D.J., Tripp R.A., Cardin R.D., Brooks J.W., Stevenson P.G. (1997) Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. Immunol. Rev. 159: 105-17.
30. Zuniga-Pflucker J.C., Jones L.A., Chin L.T., Kruisbeek A.M. (1991) CD4 and CD8 act as co-receptors during thymic selection of the T cell repertoire Semin. Immunol. 3:167-75.
31. Janeway C., & Travers P. Immunobiology. 3rd ed Singapure: Stamford Press, 1997.
32. Pescovitz M.D., Lunney J.K., & Sachs D.H. (1985) Murine anti-swine T4 and T8 monoclonal antibodies: distribution and effects on proliferative and cytotoxic T cells. J. Immunol. 134: 37-44.
33. Saalmueller A., Redheads M.J., Buhring H.J., Jonjic S., Koszinowski U.H,. (1987) Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. Eur. J. Immunol. 17: 1297-1301.
34. Yang H., and Parkhouse M. (1996) Phenotypic classification of porcine lymphocytes subpopulation in blood and lymphoid tissues. Immunology 89: 76-83.
35. Binns R.M. (1994) The null/gamma delta TCR+T cell family in the pig Vet. Immunol Immunopathol. 43: 69-77.

36. Zuckermann F.A., and Husmann R J. (1996) Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. Immunology 87: 500-512.

37. Saalmuller A, Hirt W., and Reddehase M. (1989) Phenotypic discrimination between thymic and extrathymic CD4-CD8- and CD4+CD8+ porcine T lymphocytes. Eur. J. Immunol. 19: 2011-2016.

38. Yang H., and Parkhouse M. (1997) Differential expression of CD8 epitopes amongst porcine CD8-positive functional lymphocytes subsets. Immunology 92: 45-52.

39. Zuckermann F., Pescovitz M., Aasted B., Dominguez J., Trebichavsky I., Novikov B., Valpotic I., Nielsen J., Arn S., Sachs D., Lunney J., Boyd., Walker J., Lee R., Davis W., Barbosa I., and Saalmuller A. (1998) Report on the analyses of mAb reactive with porcine CD8 for the second international. Vet. Immunol. Immunopathol. 60: 291-303.

40. Saalmuller A., Hirt W., & Redehasse M.J. (1991) Resting porcine T lymphocytes expressing class II major histocompatibility antigens. Immunobiology 183: 102-114.

41. Summerfield A., Rziha H., and Saalmueller A. (1996) Functional characterization of porcine CD4+CD8+ extrathymic T lymphocytes. Cellular Immunology 168: 291-296.

42. Saalmuller A. (1998) Antigen-specific immune respose of porcine T lymphpcytes to carios pathogens. Rev. Sci. Tech. Off. Int. Epiz. 17: 71-83.

43 Hernández J., Reyes-Leyva J., Zenteno R., Ramirez H., and Zenteno E. (1998) Immunity to porcine rubulavirus infection in adult pigs. Vet. Immunol. Immunopathol. 64: 367-381.

44. Ivanoska D., Cuperlovic K., Lunney J.K. (1990) Peripheral blood mononuclear cells subsets during *Trichinella spiralis* infection in pigs. Res Vet. Sci. 49: 92-97.

45. Abbas A.K., Williams M.E., Burstein H.J., Chang T.L., Bossu P., Lichtman A.H. (1991) Activation and functions of CD4+ T-cell subsets. Immunol. Rev. 23:5-22.

46. Mosmann T.R., Cherwinski H.M., Bond M.W., Geidlin M.A., Coofman R.L. (1986) Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136: 2348-2354.

47. Constant S.L., & Bottomly K. (1997) Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. Annu. Rev. Immunol. 15: 297-322.

48. Lederman S., Suciu-Foca N. (1999) Antigen presenting cells integrate opposing signals from CD4+ and CD8+ regulatory T lymphocytes to arbitrate the outcomes of immune responses. Hum. Immunol. 60: 533-61.

49. Harty J.T., Tvinnereim A.R., White D.W. (2000) CD8+ T cell effector mechanisms in resistance to infection. Annu. Rev. Immunol. 18: 275-308.

50. Barker E., Mackewicz C.E., & Levy J.A. (1995) Effects of TH1 and TH2 cytokines on CD8+ cell response against human immunodeficiency virus: implications for long-term survival. Proc. Natl. Acad. Sci. U. S. A. 92: 11135-9.

51. Zuckermann F.A., Zsak L., Mettenleiter T.C., Ben-Porat T. (1990) Pseudorabies virus glycoprotein gIII is a major target antigen for murine and swine virus-specific cytotoxic T lymphocytes. J. Virol. 64: 2 802-12.

52. Saalmulller A., Hirt W., Maurer S., and Weiland E., (1994) Discrimination between two subsets of porcine CD8+ cytolityc T lymphocytes by the expression of CD5 antigen. Immunology 81: 578-583

53. Saalmuller A., Asted B., Canals A., Dominguez J., Goldman T., Lunney J.K., Pauly T., Pescovitz M.D., Pospisil R R., Salmon H., and At A. (1994) Analysis of the monoclonal antibodies reactive with the porcine CD5. Vet. Immunol. Immunopathol. 43: 237-242.

54. Saalmuller A., Asted B., Canals A., Dominguez J., Goldman T., Lunney J.K., Maurer S., Pescovitz M.D., Pospisil R. R., Salmon H., and At A. (1994) Analysis of mAb reactive with porcine CD8. Vet. Immuno. Immunopathol. 43: 249-254.

55. Bradley L.M., & Swain S.L. (1998) T cell memory. Annu. Rev. Immunol. 16: 201-223.

56. Lenardo M., Ka-Ming F., Hormung F., Mc Farland H., Siegel R., Wang J., and Zheng L. (1999) Mature T lymphocytes apoptosis-immune regulation in a dynamic and unpredictable antigen environment. Annu. Rev. Immunol. 17: 221-253.

57. Murail-Krishna K., Altman J.D., Sures M., Sourdive D.J.D., Zajac A.J., Miller J.D., Slansky J., and Ahmen R. (1998) Counting antigen-specific CD8 T cells: a re-evaluation of bystander activation during viral infection. Immunity 8: 177-187

58. Jones L.A., Chin L.T., Longo D.L., and Kruisbeek M.K. (1990) Peripheral clonal elimination of functional T cells. Science 250: 1726-1729.

59. Webb S., Morris C., and Spret J. (1990) Extrathymic tolerence of mature T cells: clonal elimination as a consequence of immunity. Cell 63: 1249-1256.

60. MacDonald H.R., Baschieri S., and Lees R.K. (1991) Clonal expantion precedes anergy and death of Vβ8+ peripheral T cells responding to staphylococcal enterotoxin B in vivo. Eur. J. Immunol. 21:1963-1966.

61. Moskophidis D., Line E., and Zinkernagel R.M. (1993) Peripheral clonal deletion of antiviral memory CD8+ T cells. Eur. J. Immunol. 23: 3306-3311.

62. Tough D.F., Sun S., Zhang X., and Sprent J. (2000) Stimulation of memory T cells by cytokines. Vaccine 18: 1642-8.

63 Carter L.L., Zhang X., Dubey C., Rogers P., Tsui L., Swain S.L. (1998) Regulation of T cell subsets from naive to memory. J. Immunother. 21:181-7.

64. Zuckermann F.A., Peavey C., Schnitzlein W.M., Schabacker D., Husmann R.J., Yang H., Saalmuller A., and Lunney J.K. (1998) Definition of the specificity of monoclonal antibodies against porcine CD45 and CD45R0: report from the CD45/CD45R0 and CD44 subgroup of the Second International Swine CD Workshop. Vet. Immunol. Immunopathol. 60: 367-87.

65. Haverson K., Bailey M., Stokes C.R. (1999) T-cell populations in the pig intestinal lamina propria: memory cells with unusual phenotypic characteristics Immunology 96 :66-73.

66. Morimoto C., Letvin N.L., Boyd AW, et al., (1985) The isolation and characterization of the human helper inducer T cell subset. J. Immunol. 134: 3762-3770.

67. Ober B., Summerfield A., Mattlinger C., Wiesmuller K., Jung G., Pfaff E., Saalmuller A., and Rziha H. (1998) Vaccine-induced, pseudorabies virus-specific, extrathymic CD4+CD8+ memory t-helper cells in swine. J. Virol. 72: 4866-4873.

68. Reisner Y., Linker-Israeli M., and Sharon N. (1976) Separation of mouse thynosytes into two subpopulations by the use of peanut agglutinin. Cell. Immunol. 25: 129-134

69. Chervenak R., & Cohen J. J. (1982) Peanut lectin binding as a marker for activated T-lineage lymphocytes. Thymus 4: 61-70.

70. Verheijen F. W., Janse H. C., van Deggelen O. P., Bakker H. D., Loonen M. C. B., Durand P., and Galjaard H. (1983) Two genetically different MU-NANA* neuraminidases in human leukocytes. Biochem. Biophys. Res. Commun. 117: 470-479.

71. Taira S., & Nariuchi H. (1988) Possible role of neuraminidase in activated T cells in the recognition of allogeneic Ia. J. Immunol. 141: 440-446.

72. Lascurain R., Chavez R., Gorocica P., Perez A., Montano L.F., Zenteno E (1994) Recognition of a CD4+ mouse medullary thymocyte subpopulation by Amaranthus leucocarpus lectin. Immunology 83: 410-17.

73. Lascurain R., Porras F., Baez R., Chavez R., Martinez-Cairo S., Alvarez G., and Zenteno E. (1997) Amaranthus leucocarpus lectin recognizes human naive T cell subpopulations. Immunol. Invest 26: 579-83.

74. Pereira M. E., Kabat E. A., Lotan R., and Sharon N. (1976). Immunochemical studies on the specificity of the peanut (Arachis hypogaea) agglutinin. Carbohydr. Res. 51: 107-12.

75. Guillespie W., Paulson J.C., Kelm S., Pang M., Baum L.G. (1993) Regulation of alpha s,3-sialiltransferasa expression correlates with conversion of peanut

agglutinin (PNA+) to PNA phenotype in developing thymocytes. J. Biol. Chem. 268: 3081-4.

76. Roizman B. (1990) Multiplication of virus, an overview. In: Fields BN Knipe DM et al. (Eds) Virology, 2nd Ed., Ranven Press, New York, p87-94
77. Doring R.E., Marcil A., Chopra A., Richardson C.D.(1993) Cell 75: 295-305.
78. Kingsbury, WD. (1990) Paramixoviridae and their replication. In Fields B.N knipe M.D.Ed (1999) Virology. Raven press , New York pp: 499-961.
79. Griffin D., Ward B., and Esolen L. (1994) Pathogenesis of Measles virus infection: An hypothesis for altered immune response. J. Infect. Dis. 170(Supple 1): s24-31.
80. Doherty P. (1995) Anatomical enviroment as a determinat in viral immunity. J. Immunol. 155: 1023-1027.
81. Murray N., and McMichael A. (1992) Antigen presentation in virus infection. Curr. Opin. Immunol. 2: 361-365.
82. Martz E., & Howell D.M. (1989) CTL: virus control cells first and cytolytic cells second? DNA fragmentation, apoptosis and the prelytic halt hypothesis. Immunol Today 10, :79-86.
83. Binnendijk R., Poelen M., Kuijpers K., Osterhaus A., and Uytdehaag F. (1990) The predominance of CD8+ cells after infection with Measles virus suggest a role for CD+cell class I MHC-restricted cytotoxic T lymphocytes (CTL) in recovery from Measles. J. Immunol. 144: 2394-2399.
84. Koszinowski U., Reedhase M., and Jonjic S. (1991) The role of CD4 and CD8 cells in viral infections. Curr. Opin. Immunol. 3:471-475.
85. Biron A. C. (1994) Cytokines in the generation of immune response to, and resolution of virus infection. Curr. Opin. Immunol. 6: 530-538.
86. Schneider-Schaulies S., Kreth H.W., Hofmann G., Billeter M., and Meulen Ter V. (1991) Expression of meales virus RNA in peripheral mononuclears cells of patient with Measles, SSPE, and autoimmune diseases. Virology 182: 703-711.

87. Ray R., and Compnas R. (1990) In regenmotel M and Neurath A. (Ed) Immunochemistry for virus II. The basis for serodiagnosis and vaccines. Elseiver Science Publishers B.V. pp 217-236.

88. Reyes-Leyva J., Espinosa B., Santos G., Centeno R., Hernández J., Vallejo V., and Centeno E. (1999) Purification and Characterization of the Hemagglutinin-Neuraminidase of Porcine Rubulavirus LPMV. Glycoconjugate Journal 16: 517-522.

89. Zenteno R., Hernández J., Espinosa B., Hernandez-Jauregui P., Reyes, J., and Zenteno E. (1998) Secondary structure prediction of the hemagglutinin-neuraminidase from a porcine rubulavirus. Arch. Virol. 143: 1-20.

90. Ward F., Jhonson R., Vaisberg A., Jauregui E., and Griffin D. (1990) Spontaneus proliferation of peripheralmononuclear cells in natural Measles virus infections: Identification of dividing cells and correlation with mitogen responsivness. Clin. Immunol. and Immuniopathol. 55: 315-326.

91. Griffin D. E, Moench T. R., Johnson R. T., Lindo de Soriano I., Vaisberg A. (1986) Peripheral blood mononuclear cells during natural Measles virus Infection: cell surface phenotypes and evidence for activation. Clin. Immunol. Immunopathol. 40: 305-12.

92. Chinsakchai S. & Molitor T. (1994) Immunobiology of pseudorabies virus infection in swine. Vet. Immuno. Immunopathol. 43: 107-116.

93. Esolen L., Ward B., Moench T., and Griffin D. (1993) Infection of monocytes during Measles. J. Infect. Dis. 168: 47-52.

94. Griffin D., Ward B., and Esolen L. (1994) Pathogenesis of maesles virus infection: An hypothesis for altered immune response. J. Infect. Dis. 170(Suppl. 1): s24-31.

95. Shimizu M., Yamada S., Kawashima K., Ohashi S., Shimizu S., and Ogawa T. (1996) Changes of lymphocytes subpopulations in pigs infected with porcine reproductive and respiratory syndrome (PRRS) virus. Vet. Inmmunol. Immunopathol. 50: 19-27.

96. Ramiro-Ibáñez F., Ortega A., Ruiz-Gonzalvo F., Escribano J., and Alonso C. (1997) Modulation of immune cell populations and activation markers in the pathogenesis of African Swine Fever virus infection. Virus Research 47: 31-40.

97. Wang F., Pang V., and Hahn, E. (1988) Flow cytometric analysis of porcine peripheral blood leukosytes infected with pseudorabies virus. J. Leukoc. Biol. 43: 256-264.

98. Kimman T., Bianchi A., Wensvoort G., Bruin T., and Meliefste C. (1995) Cellular immune response to Hog Cholera virus (HCV): T cell of immune pigs proliferate in vitro upto stimulation with live HCV, but the E1 envelop glycoportein is not a major T-cell antigen. J. Virol. 67: 2922-2927.

99. Jonjic S., Mutter W., Weiland F., Reddehase M.J., Koszinowski U.H. (1989) Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4+ T lymphocytes. J. Exp. Med. 169: 1199-212.

100. Schnittman S., Lana H., Greenhouse J. Justemente J., Baseler M., and Fauci A. (1990) Preferential infection of CD4+ memory T cells by human immnuodeficiency virus type: evidence for a role in the selectine t-cell functional defects obserced in infected individuals Proc. Natl. Acad. Sci. (USA) 87: 6158-6062.

101. Page G., Wang F., and Hahn E. (1992) Interaction of pseudorabies virus with porcine peripheral blood lymphocytes. J. Leukocyte. Biol. 52: 441-448.

102. Janeway C., & Travers P. Immunobiology. 3rd ed Singapure: Stamford Press, 1997.

103. Zuckermann F., & Gaskins H.R. (1996) Distribution of porcine CD4/CD8 double-positive T lymphpcytes in mucosa-associated lymphoid tissues. Immunology 87: 493-499.

104. Croft M. (1994) Avtication of naive, memory and effector T cells. Curr. Opin. Immunol. 6: 431-437.

105. Fiorentino D.F., Bond M.W., Mosmann T.R. (1989) Two types of mouse helper T cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J. Exp. Med. 170: 2081-92.

106. Moore K.V., O'Garra A., de Waal Melafty R., Vieira P. and Mosmann T. R. (1993) Interleukin-10. Annu. Rev. Immunol. 11: 165-85.

107. Muraille E.& Leo O. (1998) Revisiting the Th1/Th2 paradigm. Scand. J. Immuno. 47:1-9.

108. Jinquan T., Larse C.G., Gesser B., Matsushima K., Thestrup-Pedersen K. (1993) Human IL-10 is a chemoattractan for CD8+, T lymphocytes an inhibitor of IL-8-inducer CD4+ T lymphocyte migration. J. Immunol. 151: 4545-4557.

109. Chen W-F. & Zlotnik A. (1991) IL-10: a novel cytotoxic T cell differentiation factor. J. Immunol. 147: 528-536.

110. Carson W.E., Lindemann M.J., Baiocchi R., et al. (1995) The functional characterization of interleukin-10 receptor expression on human natural killers cells. Blood 12: 3577-3582.

111. Levy Y. & Brouet J-C. (1994) Interleukin-10 prevents spontaneous death of germinal center B cells by induction of BCL-2 protein. J. Clin. Invest. 93: 424-430.

112. Lis H., and Sharon N. (1993). Protein glycosylation. Structural and functional aspects. Eur. J. Biochem. 218: 1-27.

113. Lis H., and Sharon N. (1989) Lectins as cell recognition molecules. Science 246: 227-34.

114. Varki A. (1993) Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 3: 97-130.

115. Alvarez G., Lascurain R., Pérez A., Degand P., Montaño L,Martínez-Cairo S., Zenteno E. (1999) Relevance of sialoglycoconjugates in murine thymocytes during maduration and selection in the thymus. Immunol. Invest. 28: 9-18.

116. Galvan M., Murali-Krishna K., Lua L., Baum L., Ahamed R. (1998) Alterations in cell surface carbohydrates on T cells from virally infected mica can distinguish effector-memory CD8+ T cells from naive cells. J. Immunol. 161: 641-648.

117. Cell surface carbohydrates in hematopoietic cell differentiation and malignancy. In: Cell surface carbohydrates and cell development. M. Fukuda (ed.) 1992. CRC Press. Boca Raton Ann Arbor London. pp. 127-159.

118. Wu W., Harley PH., Punt JA., Sharrow SO., and Kearse KP. (1996) Identification of CD8 as a peanut agglutinin (PNA) receptor molecule on immature thymocytes. J. Exp. Med. 184: 759-764.

119. Fukuda M. (1985) Cell surface glycoconjugates as onco-differentiation markers in hematopoietic cells. Biochem. Biophys. Acta 780: 119-125.

120. Zenteno E., Lascurain R., Montano L.F., Vazquez L., Debray H., Montreuil J. (1992) Specificity of Amaranthus leucocarpus lectin. Glycoconjugate 9: 204-8.

121. Sadler J. E. (1984) Biosynthesis of glycoprotein formation of O-linked oligosaccharides. In Biology of Carbohydrates V. Ginsburg, and P. W. Robbins, eds. John Wiley & Sons, New York, p. 199.

122. Landolfi N. F., Leone J., Womack J. E., and Cook R. G (1985) Activation of T lymphocytes results in an increase in H-2-encoded neuraminidase. Immunogenetics 22: 159-168.

123. Sanders M.E., Makgoba M.W., Sharrow S.O., et al., (1988) Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-1, CD2, and LFA-) and three others molecules (UCHL1, CDw29,and Pgp-1) and have enhanced INF-γ production. J. Immunol. 140: 1401-1409.

124. Mercantonio E.E., & Hynes R.O. (1988) Antibodies to the conserved cytoplasmic domain of the integrin β1 subunit react with proteins in vertebrates, invertebrates, and fungi. J. Cell. Biol. 106: 1765-1773.

125. Janeway C.A. Jr. (1992) The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. Annu. Rev. Immunol. 10: 645-74.

126. Casabo L.G., Mamalaki D., Kioussis D., and Zamoyska R. (1994) T cell activation result in physical modification of the mouse CD8β chain. J. Immunol. 152: 397-404.

127. Porras F., Lascurain R., Chavez R., Ortiz B., Hernandez P., Debray H., Zenteno E. (2000) Isolation of the receptor for Amaranthus leucocarpus lectin from murine naïve thymocytes. Glycobiology 10: 459-65.

**Datos curriculares**

Participación en congresos nacionales

1 **Hernández J**, Ramírez H, García O, Sánchez M, Vallejo V, Zenteno E y Reyes-Leyva J. (1998) "Cinética de reconocimiento inmune de proteínas virales durante la infección experimental con el rubulavirus porcino". 1a Reunión de la rama de Bioquímica y Biología molecular de Virus, Sociedad Mexicana de Bioquímica, Oaxtepec Mor.
2. Ramírez H, **Hernández J**, Mercado C, Rodríguez J, Carreón R, Borraz M, Hernández-Jáuregui P y Reyes-Leyva J. (1998) " Caracterización del rubulavirus porcino cepa PAC3 causante de alteraciones reproductivas en cerdos adultos ". 1a Reunión de la rama de Bioquímica y Biología molecular de Virus, Sociedad Mexicana de Bioquímica, Oaxtepec Mor.
3. **Hernández J**, Zenteno R, Ramírez H, Garfias Y, Espinosa B, Reyes L y Zenteno E. (1998) " Linfocitos porcinos CD4+CD8+ en la infección del rubulavirus porcino ". XII Congreso Nacional de Inmunología, Xalapa, Ver.
4. **Hernández J**, Ramírez H, Garfias Y, Reyes J y Zenteno E. (1998) "Respuesta inmune humoral y celular de cerdos infectados experimentalmente con el rubulavirus porcino". XXXIV Reunión de Investigación Pecuaria, Querétaro, Qro.
5. **Hernández J**, Ramírez H, Mercado C, Carreón R, Reyes J y Zenteno E. (1998) "Parámetros inmunológicos de una vacuna inactivada contra el rubulavirus porcino". XXXIV Reunión de Investigación Pecuaria, Querétaro, Qro.
6. **Hernández J**, Ramírez H, Zenteno R, Garfias Y, Reyes J, Hernández-Jáuregui P y Zenteno E. (1998) " Respuesta celular al rubulavirus porcino". 3a Jornada Internacional en producción porcina. Mexico D.F.
7. Zenteno R, González C, **Hernández J**, Agundis C, Huerta S, Hernández-Jáuregui P, Ramírez H y E Zenteno. (1998) "Nuevas estrategias en el diseño de vacunas contra el rubulavirus porcino (LPMV). 3a Jornada Internacional en producción porcina. Mexico D.F.
8. Ramírez H, Herrera J, Carreón R, Mercado C, Hernández P, y **Hernández J**. (1999) "Niveles hormonales en verracos inoculados con el virus de la enfermedad del ojo azul". XXXIV Congreso nacional de la Asociación Mexicana de Veterinarios Especialistas en Cerdos, Mérida, Yuc.
9. **Hernández J**, Lascuraín R, Chávez R y Zenteno E. (1999) "Marcadores fenotípicos de células de memoria en el cerdo". IV Congreso Nacional de Vacunología. México D.F.
10. Garfias Y, Jiménez C, Ortíz F, **Hernández J**, Chávez J, Chávez R, Lascuraín R, Martínez-Cairo S y Zenteno E. (1999) "Diferencias en la expresión de una estructura O-glicosilada presente en linfocitos CD4+ de pacientes pediátricos con rinosinusitis atópica en fase activa y no activa". LIII Congreso Nacional de la Sociedad Mexicana de Alergia e Inmunología, A.C. Puerto Vallarta, Mex.
11. Chávez-Rueda K, **Hernández J**, Leaños Miranda A, Blanco Favela F. (1999) "La prolactina aumenta la proliferación celular y expresión de CD154". VIII Reunión Nacional de Investigación Biomédica. Oaxtepec, Mor.

12. Espinosa S, Solis M, Garfias Y, Mercado C, Ramírez H, Zenteno E, y **Hernández J**. (2000) "Evaluación del semen de verracos inoculados con el virus de la enfermedad del ojo azul. XXXV Congreso nacional de la Asociación Mexicana de Veterinarios Especialistas en Cerdos, Acapulco Gro.
13. **Hernández J**, Mercado C, Ramírez H, Garfias Y, Reyes J, Zenteno E. (2000) "Respuesta aguda durante la infección con el rubulavirus porcino". XXXV Congreso nacional de la Asociación Mexicana de Veterinarios Especialistas en Cerdos, Acapulco Gro.
14. Mercado C, Garfias Y, Espinosa S, Solis M, Ramírez H, Zenteno E, y **Hernández J**. (2000) "Susceptibilidad in vitro de linfocitos de cerdo al rubulavirus porcino". XXXV Congreso nacional de la Asociación Mexicana de Veterinarios Especialistas en Cerdos, Acapulco Gro.
15. **Hernández J**, Mercado C, Garfias Y, Reyes J, Zenteno E. (2000) "Nuevas herramientas para evaluar los linfocitos porcinos de memoria". XXXV Congreso nacional de la Asociación Mexicana de Veterinarios Especialistas en Cerdos, Acapulco Gro.
16. Solis M, Espinosa S, Mercado C, Ramírez H, Zenteno E, y **Hernández J**. (2000) "Viabilidad del semen diluido en solución BTS, de cerdos infectados experimentalmente con el rubulavirus porcino" XXXV Congreso nacional de la Asociación Mexicana de Veterinarios Especialistas en Cerdos, Acapulco Gro

Congresos internacionales

1. B Espinosa, J Reyes, R Zenteno, **J Hernández**, H Ramírez, K Chávez and E Zenteno. (1997) "Characterization and sugar specificity of the HN from porcine rubulavirus LPM". XIV International Symposium on Glycoconjugates.
2. Gorocica P, Chávez R, Pérez A, Garfias Y, **Hernández J** y Lascurain R. (1999) "Impairement of *leismania mexicana* phagocytosis in macrophage induced by *Amaranthus leucocarpus* lectin" . FEBS, Francia.
3. **Hernández J**. (2000) "Respuesta inmune al rubulavirus porcino". Symposium internacional sobre enfermedades emergentes del cerdo. México.
4. **Hernández J**, Mercado C, Ramírez H, Lascurain R, Hernández P, Zenteno E. (2000) " PNA and ALL discriminate between memory from naive porcine lymphocytes". AAI/CIS Joint Annual Metting 2000. USA.

Publicaciones en revistas nacionales

1. **Hernández J**, Ramírez H, Zenteno R, Yonathan J, Monrroy J, Reyes-Leyva J y Zenteno. (1997) "Neumonitis inducida por el rubulavirus porcino". Revista del Inst Nal Enf Resp Mex, 10, (4): 250-255.

Publicaciones en revistas internacionales

1. Zenteno R, **Hernández J,** Espinosa B, Reyes J and Zenteno E. (1998) " Secondary structure prediction of the Hemagglutinin Neuraminidase from a porcine rubulavirus". Archives of Virology, 143: 333-352.
2. **Hernández J**, Ramírez H, Zenteno R, Reyes-Leyva J and Zenteno E. (1998) "Immunity to Porcine Rubulavirus Infection in Adult Swine". Vet Immunol and Immunopathol, 64:367-381.
3. Reyes-Leyva J, Espinosa B, Santos G, Zenteno R, **Hernández J**, Vallejo V, and Zenteno E. (1999) Purification and Characterization of the Hemagglutinin-Neuraminidase of Porcine Rubulavirus LPMV. Glycoconjugate Journal, 16: 517-522.
4. Vallejo V, Reyes-Leyva J, **Hernández J**, Ramírez H, Delannoy P, and Zenteno E. Differential expression of sialic on porcine organs during the maturation process. Comparative Biochemistry and Physiology (2000) En prensa.
5. Reyes-Leyva J., Espinosa B., Santos G., Zenteno R., **Hernández J.,** Vallejo V., Zenteno E. Purification and Characterization of the Hemagglutinin-Neuraminidase from the Porcine Rubulavirus LPMV Glycoconjugate Journal 16 (1999) 517-522.
6. **Hernández J**, Garfias Y, Nieto A, Mercado C, and Zenteno E. "Comparative evaluation of CD4+CD8- and CD4+CD8+ in the immune response to porcine rubulavirus". En preparación.
7. **Hernández J**, Garfias Y, Ramírez H, Reyes J, and Zenteno E. " Peanut agglutinin (PNA) and Amaranthus leucocarpus (ALL) lectins discriminate between memory and naive porcine lymphocytes". En preparación.

Capítulos en libros

1. **Hernández J** y Ramírez H. (1998) "Características de la respuesta inmune del cerdo". En Inmunología Veterinaria, editado por J Montaño. Ed. Ciencia y Cultura Latinoamericana, S.A. de C.V. En prensa.

Actividades académicas

1. Profesor de Asignatura, impartiendo la materia de Inmunología en la Facultad de Medicina de la UNAM, de Enero de 1995 a agosto del 2000.
2. Curso de actualización en Inmunología para médicos, impartido en el Hospital "Castro Villagrana", México D.F., Octubre de 1997. Profesor invitado.
3. "Revisión de la práctica profesional en el laboratorio de inmunología clínica". Asociación Mexicana de Bioquímica Clínica, A.C. Noviembre de 1997, duración 20 horas, Profesor invitado.
4. Curso de capacitación: "Manejo de las técnicas inmunológicas para evaluar la respuesta inmune de cerdos". Impartido en Centro de Investigación en Alimentación y Desarrollo, A. C., (CIAD, A. C.), Hermosillo, Sonora. 1-23 de Diciembre de 1997. **Profesor titular**.

5. Curso Avanzado pre-congreso del XXI-Congreso Nacional de Química Clínica. " Técnicas básicas de biología molecular y su aplicación clínica ". Marzo-1998. Profesor invitado.
6. Temas Selectos en Inmunología: "Inmunidad mediada por efectos celulares. Linfocitos T. Citotoxicidad. Mediadores y Receptores". Posgrado en Microbiología del CIM-ICUAP, BUAP. Agosto-1998. Profesor Invitado.
7. Curso de actualización en Inmunología para médicos, impartido en el Hospital "Castro Villagrana", México D.F., Octubre de 1998. Profesor invitado.
8. "Microbiología y Bioquímica aplicada a la Neumología". Escuela Nacional de Enfermería Y Obstetricia, UNAM. Noviembre-1998. Profesor invitado.
9. Mecanismos de respuesta inmune a virus. Impartido en el Posgrado de la Facultad de Medicina Veterinaria y Zootecnia de la UNAM. Febrero-Agosto de 1999. **Profesor Titular**.
10. "Diplomado en Inmunología". Facultad de Medicina, BUAP. Agosto del 2000. Profesor invitado.

Distinciones.

1. Becario de CONACyT 1994-2000.
2. Miembro del Sistema Nacional de Investigadores (Candidato a Investigador Nacional).
3. Profesor de Inmunología en el Programa de Maestría, del Posgrado de la Facultad de Medicina Veterinaria y Zootecnia, UNAM. Junio-1998.