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"ESTUDIO DE LA PATOLOGÍA Y PATOGENIA RELACIONADA CON LA RESPUESTA INMUNE INDIVIDUALY EL AGENTE ETIOLÓGICO EN LA FORMA NERVIOSA DEL VISNA/MAEDI OVINO. PROPUESTA DE UN MODELO DE CONTROL DE LA INFECCIÓN EN REBAÑOS DE ALTA PREVALENCIA."

Memoria que presenta para optar al grado de Doctor

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Directores:
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“STUDY OF PATHOLOGY AND PATHOGENY IN RELATION TO HOST INMUNE RESPONSE AND VIRUS IN NERVOUS FORM OF VISNA/MAEDI IN SHEEP. CONTROL PROGRAMME PROPOSAL OF VISNA/MAEDI INFECTION IN FLOCKS WITH HIGH PREVALENCE VALUES”

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ABREVIATURAS

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ABC: Complejo avidin-biotina peroxidasa/ Avidin-biotin peroxidase complex

ADN/DNA: Acido dexosirribonucleico/ Deoxyribonucleic Acid.

ARN/RNA: Acido ribonucleico/ Ribonucleic Acid.

BHE/BBB: Barrera hematoencefálica/ Blood brain barrier

ELISA: Ensayo de inmunoabsorbancia enzimática/ Enzyme-Linked ImmunoSorbent Assay.

IDGA: Inmunodifusión en gel agar.

IFN-γ: Interferón-gamma.

IHC: Inmunohistoquímica/ Immunohistochemistry.

IL: Interleucina/ Interleukin.

LVPR/SRLV: Lentivirus de los pequeños rumiantes/Small Ruminant Lentivirus Group.

MHC-II: Complejo mayor de histocompatibilidad de tipo II/ Major histocompatibility complex.

PCR: Reacción en cadena de la polimerasa/ Polymerase chain reaction.

SNC/CNS: Siestema Nervioso Central/ Central Nervous System.

SU: Envoltura externa.

TM: Glicoproteína transmenbranal.

VAEC/CAEV: Virus de la Artritis Encefalitis Caprina/ Caprine Arthritis Encephalitis.

VIF/FIV: Virus de la inmunodeficiencia felina/ Feline Immunodeficiency Virus.

VIH/HIV: Virus de la inmunodeficiencia huumana/ Human Immunodeficiency Virus.

VM: Visna/maedi.

VVM: Virus del Visna/maedi.

WB: Western Blot.

ZSF: Fijador de sales de zinc/ Zinc Salt Fixative

PLANTEAMIENTO Y OBJETIVOS.

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La ganadería ovina de alta producción lechera es uno de los recursos más importantes en el sector primario del ámbito rural, especialmente en regiones como Castilla y León con alta densidad de ganado ovino. En las últimas décadas ha experimentado un notable desarrollo, de la mano principalmente de la instauración de nuevos sistemas de producción intensiva y elevada selección genética. En nuestra región, la introducción de una nueva raza ovina como la Assaf Española, gran productora de leche, ha supuesto un cambio radical en los sistemas de explotación y en los niveles de productividad del sector, frente a la producción de las razas ovinas locales. Por el contrario, estas altas presiones de selección y la introducción de una raza foránea han supuesto un incremento en la tasa de algunas enfermedades, destacando entre las de origen infeccioso como el Visna/maedi ovino (VM). Esta enfermedad esta causada por un Retrovirus, *Lentivirus de Pequeños rumiantes* (LVPR) y supone pérdidas económicas importantes, principalmente debido a descensos en la producción lechera así como al desecho y muerte precoz de animales adultos en edad de alta producción (Peterhans *et al.*, 2004).

En España, esta enfermedad fue descrita por vez primera a comienzos de los años 80, en el País Vasco (González *et al.*, 1984) y Aragón (Lujan *et al.*, 1991), confirmándose posteriormente la infección en distintas zonas geográficas del país (Alba *et al.*, 2008; Lago *et al.*, 2012; Luján *et al.*, 1993; Sotelo, 1998). Actualmente, en España es una enfermedad muy extendida, especialmente en explotaciones lecheras de raza Assaf, donde la prevalencia de la infección es superior al 80% (Leginagoikoa *et al.*, 2006b; Sotelo, 1998). Esta gran importancia y extensión del VM se confirma también con los datos obtenidos de los animales estudiados en el Servicio de Diagnóstico Anatomopatológico de la Facultad de Veterinaria de la Universidad de León, donde esta enfermedad se sitúa como la más importante causa de mortalidad y desvieje prematuro en ganado ovino adulto en las explotaciones de producción intensiva de leche.

En el VM ovino se diferencian cuatro formas de presentación: nerviosa, mamaria, pulmonar y artrítica. La forma nerviosa o Visna, descrita por primera vez en la epidemia que tuvo lugar en Islandia a mediados del siglo pasado (Sigurdsson *et al*., 1957) es de presentación muy infrecuente en los países donde esta enfermedad infecciosa se ha

diagnosticado (De la Concha-Bermejillo, 1997). La mayoría de los casos clínicos descritos son hallazgos esporádicos, coexistiendo con las formas pulmonar y mamaria. Sin embargo, en la comunidad de Castilla y León, existen rebaños de ovino de raza Assaf con producción intensiva de leche que presentan en los últimos años un número muy elevado de casos nerviosos de Visna (Benavides *et al.*, 2006c; Gómez *et al.*, 1999). Así, se ha estimado que un 11,2 % de los animales que presentaban signos clínicos nerviosos y eran remitidos al Servicio de Diagnóstico Anatomopatológico de la Universidad de León, éstos se debían a lesiones nerviosas producidas por el VVM (Benavides *et al.*, 2009; Benavides *et al.*, 2006c). Este hecho, apoya la existencia de particularidades en la presentación de esta enfermedad en nuestra comunidad.

Todo ello hace que, a nuestro juicio, debería ser estudiada la forma nerviosa en ovino, intentado conocer cuales son los factores asociados a su aparición, constituyendo éste el **objetivo general** de esta tesis. Asimismo, el estudio de esta forma clínica resulta de interés como modelo animal de encefalitis causada por Lentivirus, muy similar a la descrita en infecciones por el *virus de la inmunodeficiencia humana* (VIH).

Los estudios sobre encefalitis causada por VM han sido escasos. Únicamente durante la epidemia islandesa, se presentaron casos con cierta asiduidad y se llevaron a cabo estudios detallados sobre esta forma de presentación (Georgsson, 1994; Georgsson *et al.*, 1976; Sigurdsson *et al.*, 1957; Sigurdsson and Palsson, 1958), siendo escasas las aportaciones sobre su patogenia. Las características de la lesión nerviosa son las de una encefalitis no purulenta (Sigurdsson *et al.*, 1957) localizada preferentemente en el tronco del encéfalo. Esta lesión ocurre en otras infecciones víricas del tejido nervioso y, aunque histológicamente presenta alguna característica específica, se hace necesaria una caracterización lesional precisa para así contribuir al correcto diagnóstico diferencial de esta forma de enfermedad. En los primeros estudios que se llevaron a cabo sobre el Visna en la región de Castilla y León (Gómez *et al*., 1999), ya se ponían de manifiesto bastantes singularidades en estos casos respecto a las lesiones y presentaciones clínicas descritas en la epizootia islandesa.

Los trabajos llevados a cabo en los últimos años en la Universidad de León, han puesto de manifiesto la existencia de una amplia variedad de lesiones entre los animales (Benavides *et al.*, 2009; Benavides *et al.*, 2007; Benavides *et al.*, 2006c), difiriendo en muchos casos de lo señalado en la epizootia islandesa. Estas diferencias residen

principalmente en la distribución anatómica de las lesiones en el sistema nervioso central, con un sistema de distribución principalmente perivascular, que sugeriría una progresión de las lesiones siguiendo una ruta vascular, en contraste con el patrón periependimario y la distribución de lesiones siguiendo dicha vía, propuesta previamente (Georgsson *et al.*, 1976). Además, se han descrito formas de presentación con afección exclusiva de la médula espinal (Benavides *et al.*, 2006a) no descritas hasta el momento, y diferentes patrones de lesión en función de las características del infiltrado inflamatorio y lesiones degenerativas asociadas (Benavides *et al.*, 2009). Estas diferencias de tropismo y de respuesta individual podrían ser factores determinantes relacionados con la propia genética del virus (Gonzalez *et al.*, 2005), con la del hospedador, así como con la expresión diferencial de determinados genes en distintos tejidos y órganos del hospedador y su asociación con la patogenia (Agnarsdottir *et al.*, 2000; Bolea *et al.*, 2006).

Así, el primero y segundo trabajos presentados en esta tesis tienen como fin un avance en la comprensión de la inmunopatogénesis nerviosa de esta enfermedad. Para ello, se plantean como **objetivo en el primer trabajo** de esta tesis el estudio detallado de las lesiones encefálicas para determinar si se podían establecer patrones lesionales característicos en cada individuo que pudieran venir determinados por estos factores. Para la caracterización de cada tipo lesional, se estudiaron las lesiones mediante inmunofenotipado celular, tratando de dilucidar si existía relación entre los tipos de lesiones y la presencia vírica tisular, relacionadas con diferentes respuestas inmunitarias individuales.

En el **segundo trabajo, como objetivo** se propuso el estudio de la migración vírica y extensión de la lesión en el sistema nervioso central, mediante la caracterización de los espacios perivasculares en los diferentes tipos de lesión.

Finalmente, y en relación con los trabajos y objetivos anteriores, se planteó como **objetivo inicial**, el desarrollo y puesta a punto de técnicas de inmunohistoquímica de caracterización inmunofenotípica celular en tejidos ovinos, no llevadas a cabo anteriormente en algunos inmunofenotipos y tejidos incluidos en parafina, siendo completamente necesaria para la realización de los objetivos mencionados. No obstante,

este trabajo no ha sido objeto por si mismo de publicación, sino que fue incluido en el primer trabajo.

Por otro lado, durante la realización de esta tesis cobró especial interés el diseño de un programa de control adecuado a las necesidades de la región, donde abundan las explotaciones con elevadas prevalencias de infección (más de un 80%). La aplicación de medidas de control adecuadas y eficientes, resulta fundamental en rebaños infectados con esta enfermedad donde por el momento no existen vacunas comerciales para frenar el contagio. Los datos tan elevados de prevalencia en estas explotaciones condicionan el uso de los diferentes métodos de control empleados previamente en España y otros países, basados principalmente en la eliminación de animales seropositivos y su progenie, reposición con seronegativos o la eliminación del encalostramiento natural, entre otros. Se debe tener en cuenta que estos planes de control se han realizado en rebaños de baja-media prevalencia (menor del 50%), pero resultan de difícil aplicación en los rebaños de altas prevalencias de nuestra región, ya que supondrían el sacrificio prácticamente masivo de todo el rebaño, la imposibilidad de dejar suficientes hembras de reposición nacidas de seronegativas y alteraciones importantes y costosas en el manejo en épocas de parideras para evitar el encalostramiento natural. Las pérdidas que de ello resultarían no son asumibles por los ganaderos por lo que deben aplicarse medidas menos drásticas aunque los resultados se obtengan de manera más paulatina. Por esto, un objetivo importante era la necesidad de diseñar y estudiar un programa de control de la enfermedad que redujera los niveles de infección sin modificar el manejo del rebaño y sin realizar inversiones costosas.

Así, que en el **tercer trabajo** que se presenta, se plantea como objetivo el estudio de un programa de control sencillo llevado a cabo en un rebaño con alta prevalencia de VM (93, 1%).

INTRODUCCIÓN. REVISIÓN BIBLIOGRÁFICA

1. DISTRIBUCIÓN GEOGRÁFICA DE LA ENFERMEDAD. SITUACIÓN ACTUAL EN ESPAÑA.

La enfermedad del Visna/maedi (VM) está ampliamente distribuida por toda la geografía mundial, especialmente en países de producción ovina, siendo endémica en muchos de ellos. El virus del Visna/maedi (VVM) fue erradicado en 1965 de Islandia tras la aplicación de estrictos programas de control de la enfermedad, y es el único país considerado libre de VM junto con Australia y Nueva Zelanda (Cutlip and Lehmkuhl, 1986; Luján *et al.*, 2001; Peterhans *et al.*, 2004; Reina *et al.*, 2009a).

En la década de los noventa, en Europa las diferencias eran marcadas, así mientras que en Islandia, Irlanda, Noruega y Suecia la enfermedad había sido erradicada o prácticamente eliminada (rebaños con menos de un 0.5% de prevalencia), en otros países como Holanda, Dinamarca, Reino Unido, Francia, Bélgica y España alcanzaban altos niveles de seropositividad en los rebaños. Esto se debía a la aplicación de programas de control eficaces en los primeros, en contraste con la aplicación de programas menos estrictos en los segundos (Badiola and González, 1990). Actualmente, según el portal de datos que ofrece la World Organization for Animal Health (OIE), únicamente en países como Irlanda, Islandia, Noruega, Finlandia, y Croacia no han sido detectados casos desde el año 2005 hasta el periodo actual. En contraposición, se considera presente la enfermedad en: Austria, Bulgaria, Chipre, Dinamarca, Estonia, Finlandia, Francia, Alemania, Hungría, Italia, Luxemburgo, Holanda, Polonia, Portugal, España, Serbia, Eslovaquia, Suecia, Suiza, Rumania y Reino Unido (Fuente: www.oie.int).

Atendiendo a la información que ofrece esta misma fuente de datos (OIE), la enfermedad está presente en el resto del mundo en Estados Unidos y Canadá, así como en América del Sur, aunque los datos sobre este continente son escasos al igual que en África y Asia. En Australia aún no ha sido descrita la aparición de la enfermedad (Fuente: www.oie.int).

En España, la enfermedad es considerada endémica, si bien los datos de prevalencia varían en función de los diferentes estudios y área geográfica estudiada. En

estudios previos a 1990, se estimó que la prevalencia tanto en el País Vasco como en el Valle de Ebro estaba en torno a un 50% (Badiola and González, 1990). Asimismo en otro estudio conjunto que abarcó Aragón, el cono sur de Navarra y la Rioja, las prevalencias oscilaron entre el 95-97,8% con respecto al porcentaje de rebaño afectados, y entre el 38,6-44,7% de prevalencia para los animales adultos de más de dos años de edad (Luján *et al.*, 1993). Más recientemente, en otro trabajo realizado en Aragón en 554 rebaños entre los años 2002-2007, se observó que todos los rebaños estaban infectados y presentaban una prevalencia media de 52, 8% (Pérez *et al.*, 2010). Actualmente en Cataluña, en un estudio llevado a cabo en 210 rebaños con diferentes sistemas de manejo y numero de animales, se comprobó que estaban infectados entre un 85-95% de los rebaños, con una media de prevalencia individual de cada rebaño de 58. 3%. En este estudio se constató que los percentiles 10 y 90 variaban entre 12, 5% y 91, 7% respectivamente, correspondiendo las mayores seroprevalencias a los rebaños de mayor tamaño (Alba *et al.*, 2008). En Galicia, en un estudio en explotaciones semi-intensivas de orientación cárnica, la prevalencia individual de cada rebaño estaba en torno al 24, 8%, con una media de 52, 6% de rebaños infectados (Lago *et al.*, 2012).

La prevalencia en el norte de España ha sido estimada en un 77%, sin embargo ésta se eleva a más de un 80% cuando consideramos únicamente rebaños lecheros en explotación intensiva principalmente de raza Assaf (Leginagoikoa *et al.*, 2006b). En concreto, en un estudio epidemiológico realizado en la provincia de León, los rebaños de explotación intensiva de raza Assaf presentaban elevadas prevalencias que llegaban a alcanzar valores de hasta un 96,8% (Sotelo, 1998).

2. AGENTE ETIOLÓGICO

2.1. Clasificación

El virus Visna/maedi (VVM), pertenece al grupo de virus denominado *Lentivirus de pequeños rumiantes* (LVPR) de la familia *Retroviridae*, donde están

englobadas todas la estirpes víricas del mismo, así como del virus de la artritis-encefalitis caprina (VAEC) (Zanoni, 1998). Dentro del género Lentivirus, se encuentran otros virus muy conocidos como el *virus de la inmunodeficiencia humana (VIH)* o el *virus de la inmunodeficiencia simia (VIS)* con los que comparte características patológicas similares (Leroux *et al.*, 2010; Petursson *et al.*, 1991; Thormar, 2005), por lo que su estudio resulta de gran interés.

Durante las últimas dos décadas, se han realizado numerosas descripciones de secuencias parciales o completas de aislados víricos caprinos y ovinos de diferentes zonas geográficas, así como estudios filogenéticos que demuestran la existencia de una transmisión vírica entre ambas especies e incluso a rumiantes salvajes (Erhouma *et al.*, 2008; Glaria *et al.*, 2009; Leroux *et al.*, 2010; Pisoni *et al.*, 2007; Shah *et al.*, 2004b). Además, el grupo de los LVPR ha sido dividido filogenéticamente en 5 genotipos designados con letras de A-E. El grupo A se trata de un grupo heterogéneo que contiene diferentes estirpes víricas del VVM, subdivididas a su vez en subgrupos A1-A7 donde se englobarían las cepas respiratorias y nerviosas detectadas en el norte de España (Glaria *et al.*, 2012). En el grupo B, menos complejo, y únicamente subdividido en B1 y B2, se encuentran la mayoría de los genotipos víricos responsables de la artritis caprina. En concreto el genotipo vírico responsable de las lesiones artríticas descritas en ovino en Aragón ha sido relacionado filogenéticamente con el subgrupo B2, demostrándose que su genoma es de tipo CAEV aunque en concreto la región de la integrasa del gen *pol* es de tipo Maedi (Glaria *et al.*, 2009). En el grupo C se encuentran las cepas noruegas aisladas en caprino y rumiantes salvajes (Gjerset *et al.*, 2009), y el grupo D contiene secuencias de ovinos Suizos y Españoles caracterizados por particularidades en el gen *pol*, siendo este último el grupo el filogenéticamente más distante. (Leroux *et al.*, 2010; Pisoni *et al.*, 2005; Reina *et al.*, 2006; Shah *et al.*, 2004a; Shah *et al.*, 2004b). El genotipo E ha sido descrito recientemente en Italia y confinado por el momento a esta área geográfica (Grego *et al.*, 2009).

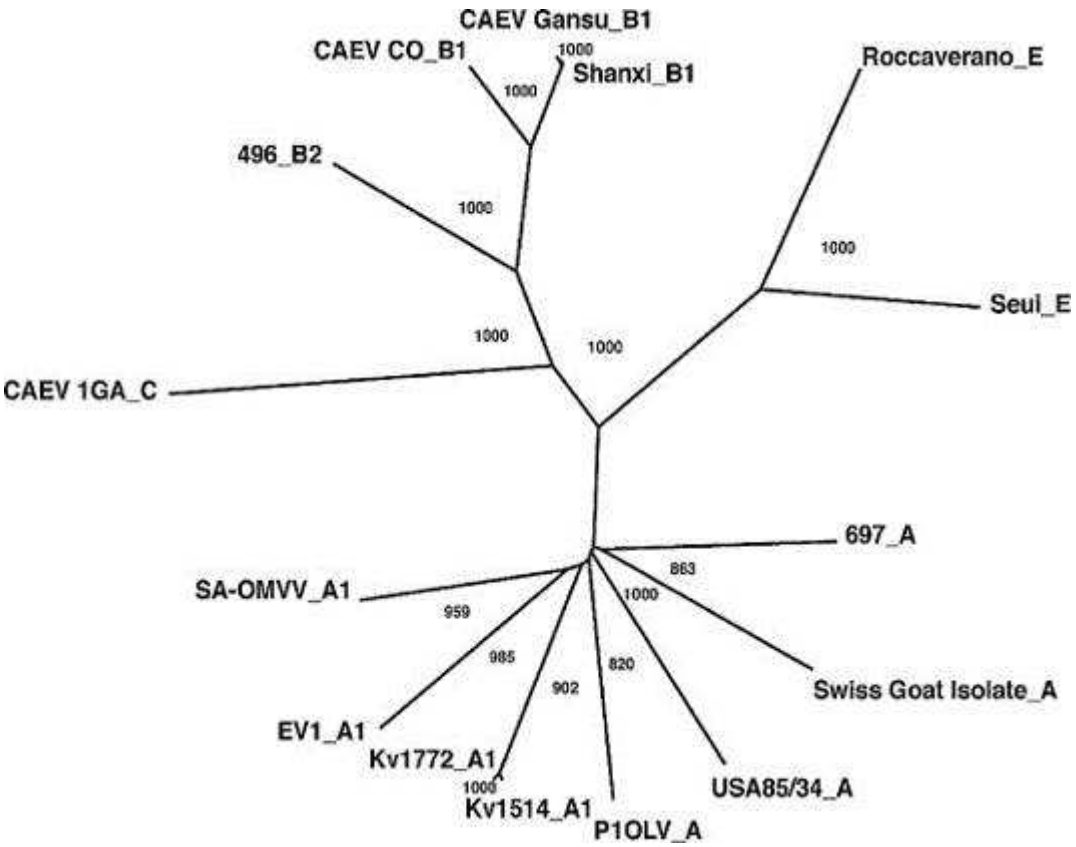

Figura 1. Arbol filogenético que comprende las secuencias de LVPR del GenBank. (Glaria 2012)

2.2. Estructura vírica

El VVM es un virus ARN cuyos viriones son partículas esféricas con envoltura, con un diámetro entre 80 y 100 nanómetros. Está constituido por una nucleocápside cónica que contiene un nucleoide denso constituido por el ácido ribonucleico (ARN) del virus, dos cadenas monocatenarias de ARN de sentido positivo, y la nucleoproteína, con enzimas como la transcriptasa inversa, la proteasa o la integrasa. La nucleocápside está rodeada de una cápside icosaédrica que posee la proteína immunogénica p25, y una matriz proteica que sirve de unión entre la cápside y la envoltura externa. A su vez, toda esta estructura está cubierta por una bicapa lipídica derivada de la célula huésped y de la inserción de glicoproteínas del virus, compuesta principalmente por dos subunidades, gp135 de superficie (SU) o gp46 (TM), que componen la envoltura externa.

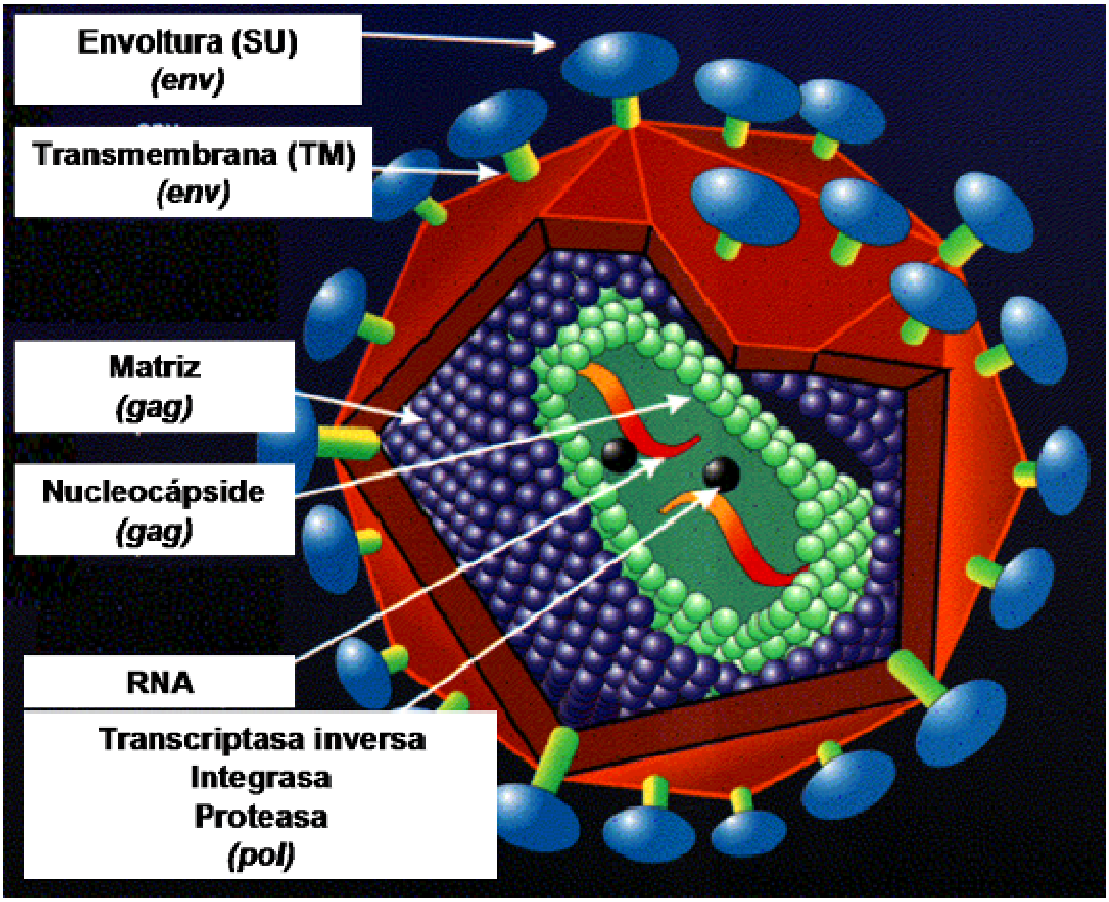

Figura 2. Estructura vírica. Imagen obtenida de: http://www.stanford.edu/~rabriggs/hiv/hiv.html

El genoma del VVM consta de 4 genes principales (5`- gag- pro- pol- env- 3`) encargados de codificar los componentes de la partícula vírica (genes estructurales), y otros tres genes reguladores (vif, rev y tat) encargados de regular la expresión de los mismos (genes reguladores). El gen *gag* codifica tres proteínas estructurales internas (especificas de grupo) de las que derivarán proteínas de la nucleocápside, de la matriz proteica y de la cápsida. En concreto el gen *gag* es el responsable de codificar la proteína p25 de la cápside que participa en la estimulación de la respuesta inmune humoral durante la infección. El gen *pro* codifica una proteasa vírica que procesa los precursores poliproteínicos. El gen *pol* contiene fundamentalmente enzimas como la transcriptasa inversa y la integrasa responsables de la integración del genoma vírico en la célula hospedadora. Por último, el gen *env* codifica las glicoproteínas de superficie, gp135 (SU) y transmembrana gp46 (TM) de la estructura proteica de superficie, participando de este modo en la asociación y entrada del virus en la célula huésped. En los extremos del ARN se encuentran dos secuencias largas de bases repetidas (LTR) que

participan en la integración del genoma vírico en el de la célula hospedadora (Leroux *et al.*, 2010; Pepin *et al.*, 1998).

3. PATOGENIA

3.1. Mecanismo de infección

Hasta el momento no se ha llegado a dilucidar si la transmisión de la enfermedad se da por la entrada de virus libre en el organismo o por la entrada de células infectadas. Es conocido que una vez que alcanza el organismo, los mecanismos de replicación viral de los lentivirus, y en concreto de LVPR, son similares a los descritos en otros retrovirus (Petursson *et al.*, 1991). La célula diana principal *in vivo* del VVM son los monocitos (Gendelman *et al.*, 1986; Gendelman *et al.*, 1985), y es a nivel tisular en el proceso de maduración de los mismos a macrófagos cuando se da principalmente el proceso de replicación vírica (Clements *et al.*, 1994; Narayan, 1983; Narayan *et al.*, 1983; Peluso *et al.*, 1985; Zhang *et al.*, 2000). Asimismo, este proceso de replicación vírica ha sido demostrado *in-vivo* en células dendríticas aferentes a ganglios linfáticos (Ryan *et al.*, 2000). Este mecanismo de infección comienza con la introducción del virus en la célula hospedadora tras la interacción de la glicoproteína vírica gp135 con la membrana celular de la célula huésped y fusión con la misma (Crane *et al.*, 1991). Una vez que la partícula vírica atraviesa la membrana plasmática, el ARN viral se libera en el citoplasma de la célula hospedadora y comienza su transcripción a una doble cadena de ácido desoxirribonucleico (ADN) por acción de la enzima transcriptasa inversa. Así, inicialmente se crea un ácido nucleico híbrido con una cadena de ARN y otra de ADN, y será a través de la transcripción de esta cadena de ADN, mediante la acción de la transcriptasa inversa actuando ahora como polimerasa, como se sintetizará la segunda cadena de ADN, para obtener un ADN de doble cadena. Este proceso de transcripción inversa es característico de los retrovirus. A continuación, este ADN proviral tiene la capacidad de fusionarse con el ADN genómico de la célula hospedadora a través de la acción de la integrasa vírica. De esta manera, la célula queda

infectada por el virus (provirus), cuyo DNA proviral queda integrado en el genoma de la misma.

Tras este proceso, el VVM reacciona de dos maneras posibles. Puede ocurrir que el virus entre en un periodo de latencia, persistiendo en las células dianas y eludiendo la respuesta inmune, o bien que el virus comience a replicarse activamente y libere viriones capaces de infectar otras células sirviéndose de los sistemas enzimáticos de la célula huésped (Zink *et al.*, 1987).

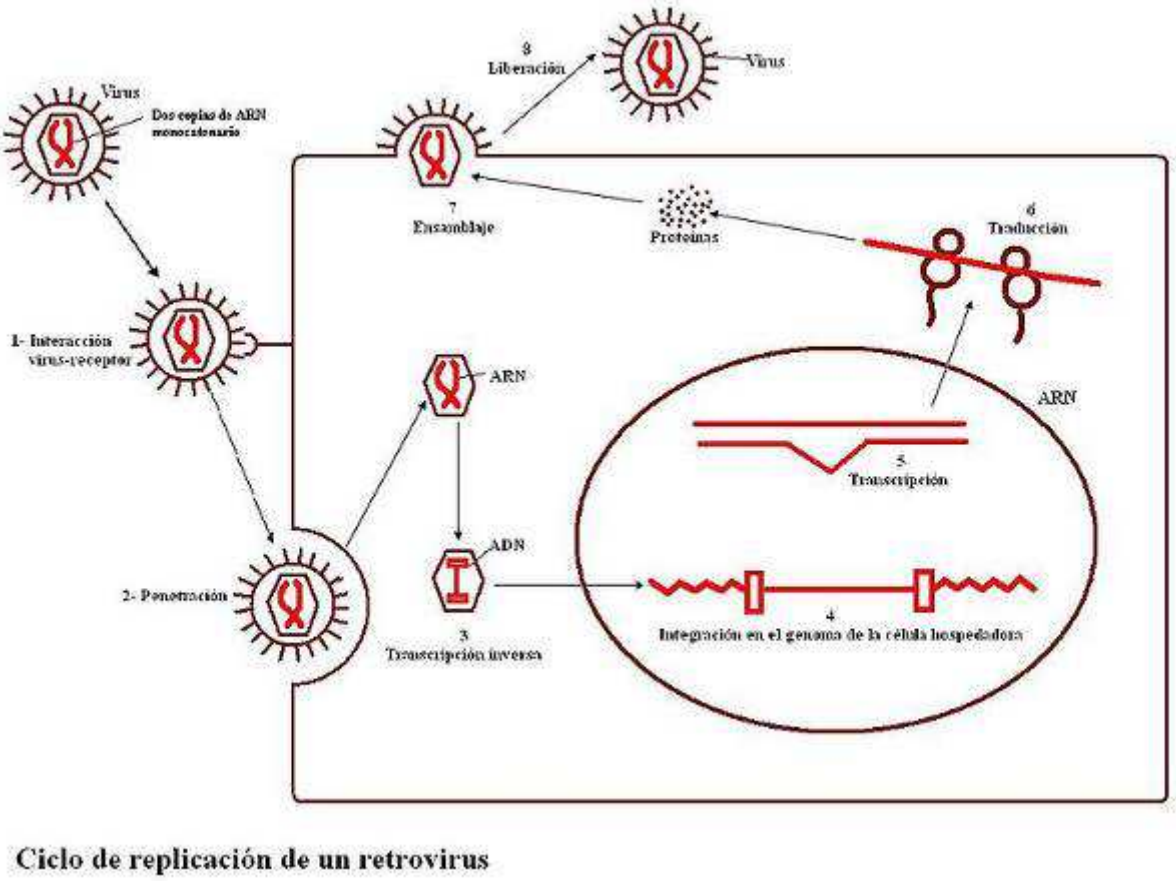

Figura 3. Ciclo biológico del virus del Maedi-Visna. Imagen procedente de: http://upload.wikimedia.org/wikipedia/commons/1/15/Ciclo_de_replicaci%C3%B3n_de_un_retrovirus.JPG

3.2. Infección y diseminación orgánica del virus

Una vez el virus penetra en el organismo se desarrollará una infección persistente donde podrán ser infectados las células dianas: macrófagos/monocitos o células dendríticas (Bird *et al.*, 1993). Se considera muy probable que a partir de estas células migre a los nódulos linfáticos regionales a partir de los que se podrá dar una diseminación sistémica (Bird *et al.*, 1993; Blacklaws *et al.*, 1995). Igualmente, se ha planteado la hipótesis de que macrófagos/monocitos infectados podrían alcanzar la médula ósea e infectar otras células precursoras de la estirpe mieloide, de forma que gracias a la maduración y multiplicación de estas células inmaduras, el propio virus vería favorecida su replicación y diseminación continua, permitiendo de esta forma el paso de células infectadas a sangre periférica de forma crónica (Blacklaws, 2012; Gendelman *et al.*, 1985).

Dado que las células infectadas no expresan proteínas víricas, éstas podrán migrar a diferentes órganos, principalmente SNC, pulmón, articulaciones o glándula mamaria, sin ser reconocidas por la respuesta inmunitaria, siendo este mecanismo de evasión conocido como "caballo de Troya" (Peluso *et al.*, 1985). El paso de estos monocitos infectados desde sangre periférica a otros tejidos propiciarán la replicación viral sirviéndose de la diferenciación de los mismos a macrófagos tisulares (Clements *et al.*, 1994; Narayan, 1983; Narayan *et al.*, 1983; Peluso *et al.*, 1985; Zhang *et al.*, 2000). Ha sido demostrado que la activación de macrófagos (fundamentalmente por citoquinas como GM-CSF, IL-1β, INF-γ, y de forma temprana por TNF-α) conduce a un incremento en la tasa de la replicación vírica, ya que existe una asociación entre la inducción enzimática de la célula huésped y la expresión vírica (Blacklaws, 2012). Como contrapunto, a nivel tisular existen mecanismos de restricción de la replicación vírica, consistentes en la limitación de la maduración de monocito a macrófago y en la alteración del ensamblaje de las partículas virales (Kennedy-Stoskopf and Narayan, 1986).

3.3. Respuesta inmunitaria

El VM se caracteriza por ser una enfermedad de progresión lenta cuya etapa subclínica puede durar meses o años, siendo la carga viral baja durante el periodo asintomático (Haase *et al.*, 1977; Pepin *et al.*, 1998; Sigurdsson and Palsson, 1958). Tanto la respuesta inmune innata como la adquirida son inducidas tras la infección por LVPR, sin embargo el virus interferirá en la correcta regulación y eficacia de la respuesta inmunitaria (Blacklaws, 2012; Reina *et al.*, 2007)

Así, una vez el virus alcanza el organismo, se producirá un pico de viremia inicial frente al que se desarrollará una intensa respuesta inmune (Pepin *et al.*, 1998). Sin embargo, la capacidad de evasión por parte del virus frente a la respuesta inmunitaria, y la ineficacia de la misma, hacen que el virus sea capaz de persistir de forma crónica en el organismo, y por lo tanto la infección lejos de ser eliminada, progresará con el tiempo y podrá llegar a manifestarse la enfermedad (Pepin *et al.*, 1998).

3.3.1 Respuesta humoral

En estudios experimentales en ovino, se ha comprobado que tras la infección se produce una intensa respuesta inmune humoral en el hospedador, donde se detectan anticuerpos específicos neutralizantes de forma variable desde los 15 días a 4 meses post-infección, hasta incluso 4 años después (Pepin *et al.*, 1998). La producción de anticuerpos es T-dependiente y se dará tras la interacción de las células presentadoras de antígeno con los linfocitos-T CD4+. Estos linfocitos T CD4+ a su vez inducirán una proliferación hacia linfocitos T cooperadores (Th, de tipo Th1 o Th2) que interactuarán con las células B para producir anticuerpos (Mariotti *et al.*, 2007; Woodall *et al.*, 1997).

Los anticuerpos se secretan frente a todas las proteínas virales, siendo los producidos frente a las proteínas de envoltura viral los que generalmente son capaces de neutralizar el virus (Blacklaws, 2012). Esta respuesta humoral es capaz de limitar la diseminación vírica aunque generalmente no produce una neutralización completa del VVM, por lo que los animales permanecen persistentemente infectados, (Lacerenza *et al.*, 2006; Pepin *et al.*, 1998). Este defecto en la respuesta inmune se ha explicado por

diferentes mecanismos: el primero, el previamente mencionado mecanismo de evasión del "caballo de Troya"; el segundo, se debería a la continua aparición de variantes antigénicas del virus (debido principalmente a mutaciones génicas en el gen *env*) cuyos epítopos no neutralizan los anticuerpos (Haflidadottir *et al.*, 2008; Narayan *et al.*, 1977a; Narayan *et al.*, 1977b). Esta alta tasa de mutación se atribuye a la actividad de la retrotranscriptasa que carece de capacidad correctora, lo que se traduce en una alta tasa de error en la transcripción vírica (Pasick, 1998); y tercero, también se ha propuesto que la alta afinidad del virus por las células diana, es decir, existe una intensa absorción del virus por parte de macrófagos/monocitos que no se ve compensada con la capacidad de las defensas humorales para neutralizar el virus (Kennedy-Stoskopf and Narayan, 1986). Teniendo en cuenta estos datos se puede señalar que la presencia de anticuerpos frente al VVM, es indicativo de infección, pero no implican niveles altos de protección.

En estudios inmunológicos de esta enfermedad ha sido demostrado que tras la infección por VVM tanto la secreción de anticuerpos neutralizantes como la fijación del complemento parece estar restringida únicamente a la subclase IgG1 de anticuerpos, siendo la secreción de IgG2 prácticamente nula. Las consecuencias a nivel patológico de este hecho aún no están claras (Bird *et al.*, 1995). En caprinos infectados con VAEC se ha relacionado la fase preclínica y clínica de la enfermedad con una intensa respuesta exclusivamente de IgG1, y en animales en fase asintomática o con infección latente con una respuesta mixta de IgG1 e IgG2, lo que podría significar que los anticuerpos IgG2 protegen frente a la manifestación de la enfermedad (Blacklaws, 2012; Trujillo *et al.*, 2004). En rumiantes, la producción de anticuerpos IgG1 es indicativa de una respuesta inmunitaria de tipo Th2, típica de animales infectados donde predomina el entorno rico en IL-4, mientras que la producción de IgG2 es indicativo de respuesta de tipo Th1, mediada especialmente por el IFN-γ y linfocitos T CD4+ (Estes and Brown, 2002).

3.3.2 Respuesta celular

Los LVPR no infectan linfocitos, a diferencia de otros lentivirus como el VIS o el VIH donde se produce una depleción de linfocitos T notable en fases clínicas

(Thormar, 2005). Sin embargo, el papel de los linfocitos, especialmente linfocitos T CD4+, en respuesta a la infección juega un papel decisivo en el desarrollo de esta enfermedad, especialmente en el control de la replicación vírica (Lee *et al.*, 1994). En infecciones víricas, los linfocitos T CD4+ participan en la producción de anticuerpos en la respuesta humoral como en el mantenimiento de los linfocitos T citotóxicos activos, lo que los convierte en imprescindibles en la respuesta inmunitaria tanto humoral como celular.

A nivel periférico, se ha constatado que una semana después de la infección, se produce una intensa activación linfocítica en respuesta al antígeno *gag*, que es capaz de controlar de forma efectiva la viremia inicial aunque no consigue eliminarla (Blacklaws *et al.*, 1995; Narayan, 1989). Se ha detectado mediante estudios de canulación *in vivo* que cuando aparece una respuesta inmune específica de linfocitos T desciende el número de células infectadas (Bird *et al.*, 1993). En concreto, la presencia a nivel periférico de un número elevado de linfocitos T citotóxico, especialmente linfocitos T CD8+, ha sido relacionada de forma indirecta en infecciones por LVPR con un descenso en los niveles víricos plasmáticos (Blacklaws, 2012; Blacklaws *et al.*, 1994; Lichtensteiger *et al.*, 1993), tal y como ha sido demostrado en otros lentivirus como VIS (Schmitz *et al.*, 1999).

A nivel tisular, una vez que las células infectadas alcanzan el tejido linfoide u órgano diana, éstas comienzan la maduración y se inicia la replicación viral sirviéndose de los mecanismos enzimáticos de la célula huésped. En consecuencia, se inducirá una respuesta inflamatoria basada en la secreción intensa de linfoquinas que estimularán la diferenciación de monocitos a macrófagos. Esta situación conducirá a una sobreexpresión del MHC-II por la continua presentación antigénica, provocando a su vez un aumento en la activación tanto de linfocitos-T como B (Torsteinsdottir *et al.*, 2007). La respuesta celular inicial estará basada principalmente en la proliferación de linfocitos-T CD8+ de carácter citotóxico, pero a pesar de que propician un control de la replicación vírica inicial, no logran alcanzar una eliminación completa de la infección (Bird *et al.*, 1993; Blacklaws *et al.*, 1994; Blacklaws *et al.*, 1995; Lujan *et al.*, 1995; Torsteinsdottir *et al.*, 1992), de forma similar a como ocurre a nivel periférico.

Esta regulación defectuosa de la respuesta inmunitaria ha sido atribuida tanto a una respuesta improductiva de los linfocitos T como a una alteración en la regulación de

la secreción de citoquinas (Tompkins and Tompkins, 2008). Los resultados de varios estudios centrados en este aspecto señalan que en animales en fase clínica de la enfermedad, los linfocitos T podrían permanecer en un estado de anergia clonal, debido a una disminución de secreción de la interleuquina-2 (IL-2) y de expresión de los receptores celulares de la misma, que conducirán a una activación crónica acompañada de ausencia de respuesta de recuerdo antígeno-específica (Begara *et al.*, 1995; Ellis and DeMartini, 1985; Mariotti *et al.*, 2007; Tompkins and Tompkins, 2008). La disminución en la respuesta celular antígeno-específica, ha sido atribuida a una menor expresión en las células presentadoras de antígeno de las moléculas coestimuladoras B7, en concreto CD80, en animales ya en fase clínica de la enfermedad (Reina *et al.*, 2007). Estas alteraciones de la respuesta inmune celular conducen a un control ineficaz de la infección viral y un desequilibrio en la regulación de la respuesta inmunitaria, donde la proliferación celular y la secreción de citoquinas se retroalimentan en un círculo vicioso, con el consecuente daño tisular que se manifestará en las lesiones características del VM (Blacklaws, 2012; Torsteinsdottir *et al.*, 2007).

Una de las citoquinas mediadoras más importantes que participan en la inmunidad celular frente a LVPR es el interferón (IFN), tanto de tipo I como de tipo II (INF-γ). En este sentido hay que tener en cuenta que las células fagocíticas infectadas no son capaces de secretar IFN por si mismas, sino que únicamente es sintetizado tras la interacción con determinados linfocitos T (Blacklaws, 2012; Woodall *et al.*, 1997). Así, el IFN tipo I es secretado en respuesta a la infección vírica y tiene un papel fundamental en la respuesta antiviral, mientras que el IFN tipo II o INF-γ es inducido tras el estímulo antigénico del virus y participa en la regulación de la respuesta inmune (Wood and Seow, 1996). De hecho, se ha demostrado que el tratamiento con IFN tipo I en corderos inmediatamente después de la infección con VVM produce una reducción efectiva de la carga viral, ya que una de las funciones del INF tipo I es la inhibición de la proliferación y diferenciación de monocitos limitando de esta forma la replicación vírica (Juste *et al.*, 2000; Kennedy *et al.*, 1985; Zink *et al.*, 1987). Sin embargo, con respecto al IFN-γ, los LVPR han desarrollado vías de escape a la acción del mismo, incluso parece que en la infección por VAEC, el IFN-γ podría llegar a jugar un papel antagónico al habitual, produciendo una activación de la replicación vírica en monocitos tras interaccionar con la región genómica LTR del virus (Tong-Starksen *et al.*, 1996)

4. TRANSMISIÓN Y VÍAS DE CONTAGIO

Experimentalmente, la infección por VVM ha conseguido ser transmitida mediante diferentes tipos de inóculos (secreciones de animales infectados, agua contaminada con los mismos, homogeneizados de órganos o aislamientos víricos), por vía respiratoria (intratraqueal, intranasal, intrapulmonar o intratorácica), intracerebral, endovenosa, digestiva, intramuscular, subcutánea, intrarticular e incluso mediante inoculación en el saco amniótico (Badiola and González, 1990; Torsteinsdottir *et al.*, 2003). Sin embargo, de forma natural se considera que las vías más importantes de transmisión de VVM entre animales son dos: la transmisión horizontal y la transmisión lactógena, cuya contribución relativa a la diseminación de la infección ha sido debatida en diferentes estudios (Berriatua *et al.*, 2003b; Blacklaws *et al.*, 2004; Leginagoikoa *et al.*, 2010; Álvarez *et al.*, 2005).

La transmisión horizontal es debida al contacto directo con animales infectados, mediante la aspiración de aerosoles u otras secreciones procedentes de órganos infectados (Blacklaws *et al.*, 2004; Luján *et al.*, 1994; Peterhans *et al.*, 2004). En este sentido se ha demostrado la presencia de virus libre en el fluido respiratorio (McNeilly *et al.*, 2007), y ha reproducido experimentalmente la enfermedad a través de la infección por vía intranasal, intratraqueal e intrapulmonar (Niesalla *et al.*, 2008; Torsteinsdottir *et al.*, 2003). Así, el contagio vía oronasal, especialmente mediante el contacto entre el cordero y su madre infectada, es considerado el modo más efectivo de transmisión de la enfermedad (Blacklaws *et al.*, 2004; Houwers *et al.*, 1989; Leginagoikoa *et al.*, 2006a; Leginagoikoa *et al.*, 2010). Recientemente ha sido demostrada la presencia de ácidos nucleicos del virus en agua de bebederos y en aire exhalado de animales infectados que podrían estar implicados en la transmisión de la enfermedad (Villoria *et al.*, 2013).

La transmisión lactógena madre-cordero por el consumo de calostro y leche procedente de madres infectadas, parece relacionarse con la entrada del virus en el organismo presumiblemente a través del tracto digestivo (Blacklaws *et al.*, 2004; Cutlip *et al.*, 1985a). Con la intención de confirmar esta vía de transmisión de la enfermedad,

han sido detectadas partículas de LVPR en tejido mamario de hembras lactantes, así como en células epiteliales mamarias y macrófagos en leche y calostro (Blacklaws *et al.*, 2004; Sihvonen, 1980). Asimismo, se ha demostrado la presencia de VVM en células mononucleares del tejido linfoide de la submucosa intestinal de corderos alimentados con calostro infectado (Preziuso *et al.*, 2004b), planteándose de esta forma la posibilidad de entrada del virus a través de esta ruta. Parece evidente que el padecimiento de la forma mamaria del VM por parte de las madres infectadas incrementaría el riesgo de transmisión materna (Houwers *et al.*, 1989; Álvarez *et al.*, 2005). En este sentido, en estudios realizados en corderos privados de consumo de calostro y alimentados de forma artificial con leche ovina pasterizada o bovina, se demostró que esta era una medida que controlaba la transmisión de la enfermedad por esta vía (Houwers *et al.*, 1983). Asimismo, se ha comprobado que las corderas nacidas de madres seronegativas tienen una menor tendencia a seroconvertir en un futuro. Este hecho se ha relacionado con la no ingestión de leche o calostro infectado, pero también con una posible mayor resistencia genética a la infección trasmitida por la madre (Blacklaws *et al.*, 2004; Leginagoikoa *et al.*, 2006a). Sin embargo, en condiciones naturales esta vía de contagio parece tener una importancia relativa menor en la diseminación de la infección en comparación con la transmisión de la infección por vía aerógena entre la madre infectada y el cordero, donde la duración del estrecho contacto entre ambos parece jugar un papel decisivo (Broughton-Neiswanger *et al.*, 2010; Leginagoikoa *et al.*, 2006b; Álvarez *et al.*, 2005).

La transmisión vertical ha sido descrita también como vía de contagio de la infección, a través de la transmisión madre-feto, aunque su importancia epidemiológica ha sido considerada muy limitada (Blacklaws *et al.*, 2004; Broughton-Neiswanger *et al.*, 2010; Cortez-Romero *et al.*, 2011; Peterson *et al.*, 2008; Romero *et al.*, 2010; Álvarez *et al.*, 2006). En concreto, en el aparato genital femenino ha sido identificado el VVM en ovario, oviducto y útero (Cortez-Romero *et al.*, 2011), y aunque se ha confirmado que en el ovario, las células de la granulosa pueden albergar el virus, las células de los folículos ováricos y ovocitos permanecen libres del VVM (Cortez Romero *et al.*, 2006). La zona pelúcida del embrión parece ser igualmente una barrera efícaz frente a la infección por el VVM (Romero *et al.*, 2010). Por ello, la transmisión vertical es considerada de escasa relevancia epidemiológica, y si bien se han confirmado casos de forma muy ocasional (Blacklaws *et al.*, 2004). Sin embargo, otros estudios han obtenido

resultados que cuestionan este dato, ya que observan que entre un 5-8% de los animales recién nacidos de madres seropositivas resultan ser LTR-PCR positivos en sangre al nacimiento (Álvarez *et al.*, 2006). También se ha propuesto la vía de contagio seminal a través del macho. Mediante el uso de PCR se ha detectado DNA proviral en los testículos, epidídimo y en todas las glándulas sexuales accesorias (Peterson *et al.*, 2008). Sin embargo, se ha observado que el semen eyaculado presenta una secreción discontinua de DNA proviral (Peterson *et al.*, 2008), especialmente acusada en presencia de otras coinfecciones como por *Brucella. ovis*, hecho que podría constituir un factor de riesgo de infección tanto para la hembra como para el cordero (Preziuso *et al.*, 2003a). En un estudio reciente, se ha evidenciado la capacidad de infección virica por VAEC a través de la inseminación artificial con semen infectado en cabras seronegativas. Los resultados concluyen que la infección seminal es posible dado que 12 cabras de 20 inseminadas seroconvierten 60 días post-inseminación (Souza *et al.*, 2013).

5. MANIFESTACIÓN CLÍNICA DE ENFERMEDAD, LESIONES Y DIAGNÓSTICO ANATOMOPATOLÓGICO.

La manifestación clínica de la enfermedad del Visna/maedi se da principalmente en ovinos ya en edad adulta dada la progresión lenta de la enfermedad, aunque la forma nerviosa se ha descrito en animales de tan solo 4-6 meses (Benavides *et al.*, 2007). Clínicamente se caracteriza por ser un síndrome multisistémico que cursa con una pérdida progresiva de la condición corporal, y que causa inflamación crónica no purulenta de los pulmones, glándula mamaria, articulaciones y SNC (Cutlip *et al.*, 1979; Cutlip *et al.*, 1985a; Cutlip *et al.*, 1988; Dawson, 1987; Sigurdsson *et al.*, 1957). En ovino, la forma de presentación más frecuente del VM es la forma respiratoria y mamaria, mientras que la forma nerviosa y articular se manifiestan de forma más esporádica (Benavides *et al.*, 2009; Benavides *et al.*, 2006c; Cutlip *et al.*, 1979; Gómez *et al.*, 1999; Lujan *et al.*, 1991; Sigurdsson *et al.*, 1957). Sin embargo, en Castilla y León desde hace varios años se han diagnosticado frecuentemente lesiones de encefalitis asociada a VM, y se ha estimado que un 11,2 % de los animales remitidos al

Servicio de Diagnóstico Anatomopatológico de la Facultad de Veterinaria de la Universidad de León que presentaban signos clínicos nerviosos, éstos se debían a lesiones nerviosas producidas por el VVM (Benavides *et al.*, 2009; Benavides *et al.*, 2006c). Las estirpes de VM neurotrópicas, en concreto las cepas aisladas en España están englobadas en el genotipo A, de forma que su filogenia resulta muy similar a las cepas respiratorias (Glaria *et al.*, 2009; Ramirez *et al.*, 2012). Asimismo, se han descrito recientemente numerosos casos de VM que se manifiestan con la forma articular de la enfermedad en la Comunidad Autónoma de Aragón (Pérez *et al.*, 2012), caracterizada por una artritis crónica proliferativa producida por cepas víricas de LVPR de tipo AEC. Así, la estirpe vírica española responsable de las lesiones articulares ha sido englobada en el genotipo B, al que pertenecen numerosas cepas de AEC (Glaria *et al.*, 2009).

Las lesiones que se desarrollan en esta enfermedad se caracterizan por una infiltración de macrófagos, linfocitos, células plasmáticas, en los órganos diana que gradualmente serán los responsables de la manifestación clínica de la enfermedad. Además, este infiltrado inflamatorio puede acompañarse de hiperplasia de tejido linfoide con formación de folículos linfoides germinales (Cordier *et al.*, 1992; Georgsson *et al.*, 1976; Lujan *et al.*, 1991; Palsson, 1976; Torsteinsdottir *et al.*, 1992; van der Molen *et al.*, 1985).

5.1. Forma respiratoria

Los animales que presentan la forma pulmonar del VM, inicialmente presentan una ligera apatía, y menor ganancia de peso que podrá progresar a dificultad respiratoria (disnea) acompañado de pérdida de condición corporal (Dawson, 1987). En el momento que la gravedad de los signos respiratorios aumenta, los animales afectados se reconocen en el campo por la intolerancia al esfuerzo físico (De Boer *et al.*, 1979). La manifestación clínica de dificultad respiratoria se debe a una neumonía intersticial crónica no purulenta que dificultará el intercambio normal de gases (Georgsson and Palsson, 1971).

Al realizar la necropsia de ovinos con las manifestaciones clínicas respiratorias de VM, los pulmones afectados no se observan colapsados y presentan macroscópicamente un evidente incremento de volumen y de peso (mayor de 700 g), acompañados de una consistencia gomosa y una coloración grisácea (Cutlip *et al.*, 1988; Dawson, 1980; Lujan *et al.*, 1991; Sigurdsson *et al.*, 1952). Además los nódulos linfáticos regionales (traqueobronquiales y mediastínicos) aparecen muy aumentados de tamaño y de color blanquecino (Badiola and González, 1990).

Estas alteraciones macroscópicas pulmonares, se deben a la presencia de un infiltrado inflamatorio intersticial de células mononucleares (principalmente linfocitos, macrófagos y células plasmáticas) en las paredes alveolares que se traducen en una neumonía intersticial crónica no purulenta (Cutlip *et al.*, 1988; Georgsson *et al.*, 1989; Sigurdsson *et al.*, 1952). Asimismo, estos cambios suelen acompañarse de una hiperplasia linfoide difusa consistente en agregados linfoides distribuidos por todo el parénquima pulmonar, localizados generalmente alrededor de bronquiolos y vasos sanguíneos llegando a formar folículos linfoides (Palsson, 1976), que ocasionalmente pueden ser observados macroscópicamente como un punteado grisáceo subpleural de 0, 5-1 mm (Cutlip *et al.*, 1988; Lujan *et al.*, 1991). Generalmente estos cambios se acompañan de una hiperplasia evidente de las fibras musculares lisas, así como de una proliferación de fibroblastos y depósito de colágeno responsables del aumento de consistencia (Badiola and González, 1990).

El número de linfocitos T CD4+ y CD8+ está aumentado en los tabiques interalveolares (Watt *et al.*, 1992b), presentando una proporción similar de CD4+/CD8+ en animales en fase asintomática, y con predominio de linfocitos-T CD8+ en animales ya con signos clínicos respiratorios (Cadore *et al.*, 1996; Cordier *et al.*, 1992).

5.2. Forma mamaria

La presentación clínica de la forma mamaria del VM consiste en una progresiva atrofia e induración de ambas glándulas mamarias (Cutlip *et al.*, 1985a; Houwers *et al.*, 1988; Lujan *et al.*, 1991; Pekelder *et al.*, 1994; van der Molen and Houwers, 1987; Watt

et al., 1992a). La leche conserva su aspecto normal si bien se puede producir un descenso acusado de producción láctea e incluso agalaxia (Dawson, 1987).

Las glándulas mamarias afectadas presentan un incremento difuso de consistencia sin que se manifiesten generalmente otros cambios macroscópicos. Los nódulos linfáticos regionales retromamarios suelen observarse notablemente incrementados de tamaño (Badiola and González, 1990).

Histológicamente, en el parénquima mamario afectado se observa un infiltrado inflamatorio de predominio linfocítico dispuesto entre túbulos y alvéolos mamarios y alrededor de conductos galactóforos (Lujan *et al.*, 1991; van der Molen and Houwers, 1987; van der Molen *et al.*, 1985). Igualmente, se observa degeneración de células secretoras y alteración de la estructura túbulo-alveolar normal de la glándula (Luján *et al.*, 2001). Asimismo, en relación con las unidades secretoras y de forma periductal es común observar agregados/folículos linfoides y abundante tejido conjuntivo fibroso puede provocar la obliteración de conductos galactóforos (Houwers *et al.*, 1988; van der Molen *et al.*, 1985).

Asimismo, se ha demostrado la presencia de macrófagos infectados por el virus en el tejido intersticial, que parecen ser los responsables de estimular una respuesta inmune inicial, local y difusa por el parénquima mamario que se traducirá en la mamitis intersticial difusa crónica no purulenta característica de la forma mamaria de esta enfermedad (Cutlip *et al.*, 1985a).

5.3. Forma articular

Las lesiones articulares se manifiestan de forma muy similar a las producidas por el VAEC. Así, clínicamente se caracteriza por una tumefacción de las articulaciones, generalmente del tarso y carpo, debido a una sinovitis proliferativa acompañada de fibrosis y calcificación de los tejidos blandos que provocan una cojera progresiva del animal junto con necrosis y degeneración del cartílago articular (Cutlip *et al.*, 1985b; Pérez *et al.*, 2012). Histológicamente, esta lesión crónica se caracteriza por

una infiltración masiva de la sinovia por células mononucleares y por la presencia agregados y folículos linfoides (Cutlip *et al.*, 1985b). La caracterización celular por inmunohistoquímica se ha realizado en AEC pero no en VM. Experimentalmente se ha comprobado que en lesiones iniciales, día 12 post-infección, el infiltrado inflamatorio esta compuesto primeramente por linfocitos T CD8+, mientras que las células T CD4+ van incrementando su número con el paso de los días (von Bodungen *et al.*, 1998). A los 33 días post-infección, las células B y macrófagos ven incrementado su número de forma muy evidente en detrimento de los linfocitos (von Bodungen *et al.*, 1998; Wilkerson *et al.*, 1995).

5.4. Forma nerviosa

En el brote Islandés, la forma de presentación nerviosa de la enfermedad del VM fue la más representativa, sin embargo actualmente esta forma de afectación se considera esporádica (Benavides *et al.*, 2006a; Benavides *et al.*, 2009; Lujan *et al.*, 1991; Sigurdsson *et al.*, 1957). Sin embargo se han detectado numerosos casos de encefalitis en Castilla y León en ovinos de raza Assaf en producción intensiva lechera, como hemos señalado anteriormente en la introducción de este apartado.

Generalmente, la manifestación clínica ocurre en animales adultos por encima de dos años, pero ya ha sido diagnosticada en corderos de de 4 y 6 meses de vida (Benavides *et al.*, 2007). Los principales signos clínicos que se observan es estos animales son: debilidad de las extremidades, ataxia, decúbito y postración, permaneciendo el animal alerta y atento a estímulos externos (Benavides *et al.*, 2009; Benavides *et al.*, 2006c; Christodoulopoulos, 2006; Sigurdsson *et al.*, 1957).

Macroscópicamente, las lesiones en el encéfalo y en la médula espinal pueden ser inaparentes, pero es posible observar áreas de malacia en la sustancia blanca del encéfalo (principalmente en áreas periventriculares y en puente y pedúnculos cerebelosos), y zonas en forma de cuña de necrosis por licuefacción en la sustancia blanca de secciones transversales de la médula (Benavides *et al.*, 2006a; Benavides *et*

al., 2009; Sigurdsson *et al.*, 1957; Sigurdsson and Palsson, 1958). Histológicamente, la lesión más característica es la presencia de amplias zonas de desmielinización y meningoencefalitis no purulenta. El infiltrado inflamatorio que se observa está formado principalmente por células mononucleares (linfocitos, macrófagos y células plasmáticas), siendo característica la presencia de células "gitter", macrófagos de citoplasma abundante, claro y espumoso encargados de fagocitar la mielina (Benavides *et al.*, 2009; Benavides *et al.*, 2006c; Gómez *et al.*, 1999). Los plexos coroideos suelen presentar asimismo presentar un infiltrado inflamatorio no purulento de células mononucleares que se disponen formando agregados o folículos linfoides (Cutlip *et al.*, 1979). Estas lesiones histológicas se han sido clasificado teniendo en cuenta la localización de la lesión y la población del infiltrado celular observado: Tipo *Vascular*, donde las células inflamatorias se disponen formando manguitos perivasculares; *tipo infiltrativo*, donde además se observa en el neuroparénquima abundante infiltrado inflamatorio; y *tipo malácico*, donde la característica principal es la presencia de abundante desmielinización (Benavides *et al.*, 2009).

En la lesión nerviosa se ha demostrado que el papel de la inmunidad celular, resulta fundamental en el desarrollo de la encefalitis (Torsteinsdottir *et al*, 1992). Aunque aún no se conoce completamente el mecanismo patogénico se ha propuesto como hipótesis que la infección vírica del neuroparénquima propiciaría la migración de células inflamatorias al neuroparénquima. Esto conllevaría un incremento en la secreción de citoquinas, que a su vez actuarían como sustancias quimiotácticas para estas células (Craig *et al.*, 1997; Ebrahimi *et al.*, 2000; Georgsson, 1994; Georgsson *et al.*, 1976; Torsteinsdottir *et al.*, 2007). En un estudio previo realizado en un único animal que presentaba lesiones nerviosas de VM, se comprobó que el infiltrado inflamatorio del neuroparénquima presentaba una proporción de linfocitos CD4/CD8 de 0.8, y en los espacios perivasculares un 10% de las células eran monocitos/macrófagos y la proporción de linfocitos CD4+/CD8+ era de 1/3 (Torsteinsdottir *et al*, 1992), de forma similar a como ocurre con otros lentivirus (Georgsson, 1994).

6. DIAGNÓSTICO

Dado que no existen vacunas comerciales disponibles, el establecimiento de un programa adecuado de diagnóstico y control en un rebaño, resultará indispensable para controlar la infección por el VVM (Peterhans *et al.*, 2004). La mayoría de estas estrategias de control están apoyadas en métodos de diagnóstico *in vivo* que permiten la diferenciación entre animales infectados y libres de infección. Estos métodos pueden agruparse en dos: métodos de detección indirectos (determinación de anticuerpos en sangre frente al VVM) y métodos de detección directos del virus (pruebas moleculares de detección de proteínas y ácidos nucleicos virales).

6.1. Métodos de detección indirectos o serológicos.

Tal y como se ha mencionado previamente, en las infecciones por LVPR los animales seroconvierten desde las 2 semanas hasta los 4 meses post-infección, y generalmente suelen mantener la seropositividad de forma permanente, de modo que la detección de anticuerpos resulta un instrumento muy útil para identificar un porcentaje mayoritario de los animales infectados (Lacerenza *et al.*, 2006; Pepin *et al.*, 1998). Se considera, tras la realización de estas pruebas, que los animales con resultado seropositivo son portadores del virus. Sin embargo, siempre hay que tener en cuenta los animales recientemente infectados del rebaño que todavía no han seroconvertido o que tienen un título de anticuerpos aún muy bajo, cuyos resultados serológicos pueden ser negativos a pesar de la infección.

Las pruebas serológicas más comúnmente utilizadas y propuestas por la OIE como pruebas “prescritas” para el comercio internacional de animales son la inmunodifusión en gel de agar (IDGA) y la pruebas serológicas de enzimoinmunoensayo (ELISA) (OIE, 2008). También existen pruebas inmunológicas específicas como Western-blot, radioinmunoensayo o radioinmunoprecipitación, pero el uso de éstas es menos común, y generalmente se realizan de forma más especializada, en laboratorios con fines concretos principalmente para confirmación del diagnóstico.

6.1.1 Pruebas serológicas tipo ELISA

Se trata de una técnica económica y la más ampliamente utilizada hoy en día, de la que existen kits serológicos comerciales con buenas características de especificidad y de sensibilidad. La sensibilidad y especificidad depende en gran medida de la homogeneidad entre el antígeno comercial presente en el kit, y el genotipo de VVM frente al que se han desarrollado los anticuerpos. De forma que si el antígeno empleado en el kit presenta grandes diferencias con el genotipo infectante, los anticuerpos del suero problema podrían no conjugarse con el antígeno, dando como resultado falsos negativos, como ha sido detectado en animales infectados por el genotipo E (Reina *et al.*, 2009b). Existen tres tipos de kits ELISA disponibles cuya diferencia se encuentra en el antígeno que va ligado a la placa: este antígeno puede tratarse del virus completo purificado y obtenido de sobrenadantes de cultivos celulares, o bien de proteínas o péptidos recombinantes obtenidos mediante técnicas de recombinación con plásmidos en cultivos de *Escherichia coli*. El antígeno que proporcionaría a la técnica ELISA mayor sensibilidad sería una combinación de un antígeno del núcleo y un antígeno de la envoltura vírica (De Andrés *et al.*, 2005). Se ha demostrado que es posible detectar anticuerpos frente al VVM en tanques de leche, para el diagnóstico rápido y de bajo coste de infección en un rebaño, tanto en ovino como caprino (Brinkhof *et al.*, 2010a).

La técnica de diagnóstico ELISA exige un equipamiento especializado de los laboratorios (reactivos y espectofotómetro), siendo la más adecuada para los diagnósticos a gran escala. En concreto, la sensibilidad y especificidad del test ELISA (Elitest®, Hyphen BioMed, Neuville/Oise, Francia) utilizado en este estudio han sido estimadas en 99, 4% y 99,3%, respectivamente (De Andrés *et al.*, 2005; Saman *et al.*, 1999). El antígeno recombinante utilizado es un péptido inmunogénico de la glicoproteína transmembrana gp46 y la proteína recombinante p25.

6.1.2. Pruebas serológicas tipo IDGA.

La IGDA es una prueba fácil de realizar y de alta especificidad, sin embargo el hecho de que la sensibilidad de la técnica no sea tan alta como el ELISA (Celer *et al.*, 1998), unido a su difícil automatización y la subjetividad en la interpretación de los resultados, hacen que esta técnica no sea la más utilizada actualmente (De Andrés *et al.*, 2005). Además, es capaz de detectar la seroconversión de una manera menos precoz que el test ELISA, por lo que animales recientemente infectados seropositivos se detectan con mayor dificultad (Lujan *et al.*, 1991; Saman *et al.*, 1999; Varea *et al.*, 2001). En un estudio realizado en 2001, se estimó una sensibilidad y especificidad de la técnica de 76, 3% y 98, 3% respectivamente (Varea *et al.*, 2001). Estas pruebas serológicas han sido desarrolladas frente a la glicoproteína de la envoltura vírica denominada gp135 (VM) y frente a la proteína interna p25 (p28 en AEC). La prueba serológica consiste en la difusión, a través de un gel de agarosa, del suero problema y el antígeno, con sueros control que determinarán la presencia o ausencia de anticuerpos anti-VVM en el suero problema. En este tipo de prueba serológica, el uso de un antígeno adecuado también resulta muy importante para que la unión epítopo–anticuerpo sea eficaz y no obtener falsos negativos.

6.2 Detección de proteínas y ácidos nucleicos virales.

Mediante métodos de diagnóstico directo como técnicas moleculares, inmunohistoquímicas o técnicas de co-cultivo es posible reconocer secuencias específicas del VVM u obtener un aislamiento vírico, sin embargo, aunque resulta una herramienta útil para la confirmación de un diagnóstico o evidenciar animales infectados seronegativos, no es una técnica de diagnóstico adecuada para utilizar a gran escala.

Algunas de estas pruebas como la Reacción en Cadena de la Polimerasa (PCR) resultan el complemento ideal a la serología en programas de erradicación de la enfermedad, ya que estos métodos son capaces de detectar animales recientemente infectados que aún no han seroconvertido. La PCR convencional es capaz de detectar

directamente ácidos nucleicos del ADN del virus en forma de provirus integrado en la células infectantes, o bien del ARN del virión tras un proceso de retrotranscripción, donde este ARN de transformará en ADN tras la acción de una retrotranscriptasa inversa (RT) (Extramiana *et al.*, 2002). Sin embargo, esta técnica se encuentra limitada por la baja carga viral de la muestra, por lo que han aparecido otras variantes como la PCR anidada (Capucchio *et al.*, 2003; Preziuso *et al.*, 2003b) o la PCR cuantitativa a tiempo real (Carrozza *et al.*, 2010; Herrmann-Hoesing *et al.*, 2007) que consiguen una mayor sensibilidad y especificidad. También puede determinar la presencia vírica mediante la detección en sangre de ADN proviral, sin embargo la carga viral a nivel periférico es mucho menor que en los tejidos diana, y no siempre detectable (Leginagoikoa *et al.*, 2009). Otro problema de esta técnica se basa en la variabilidad genética del virus, que hace imprescindible una buena elección de cebadores para la correcta amplificación de la secuencia vírica que buscamos, generalmente LTR, *gag*, *pol y env* (Carrozza *et al.*, 2010; Extramiana *et al.*, 2002). Para evitar este problema, diversos autores indican que la secuencia de LTR es la que presenta mayor conservación (Extramiana *et al.*, 2002).

La técnica de PCR se ha utilizado para la detección del virus en sangre, en coágulo de sangre, leche, semen y otros tejidos animales como tercer párpado (Angelopoulou *et al.*, 2006; Barquero *et al.*, 2011; Brinkhof *et al.*, 2010a; Capucchio *et al.*, 2003; Extramiana *et al.*, 2002; Herrmann-Hoesing *et al.*, 2007; Leginagoikoa *et al.*, 2009; Peterson *et al.*, 2008; Preziuso *et al.*, 2003a; Álvarez *et al.*, 2006). Además, esta técnica resulta muy útil para confirmación del diagnóstico en estudios anatomopatológicos, ya que la PCR es posible realizarla a partir de tejido en parafina, siempre y cuando el tiempo de fijación de la muestra no sobrepase las 48 h, ya que los líquidos fijadores fragmentan el DNA vírico impidiendo su amplificación (Benavides *et al.*, 2006b).

Otra técnica alternativa, y que puede resultar necesaria para determinados estudios, es el aislamiento vírico. El aislamiento de virus del propio animal se puede realizar co–cultivando células del animal de tejidos o de fluidos corporales del animal con cultivos celulares de ovino adecuados, como células endoteliales del plexo coroideo para luego valorar el efecto citopático (Torsteinsdóttir *et al.*, 1997). Sin embargo, esta técnica resulta lenta, laboriosa y no siempre eficaz.

La combinación de uno o más métodos de diagnóstico, principalmente test ELISA y técnicas PCR, parecen ser la solución óptima para evitar errores de diagnóstico de infección (Brinkhof *et al.*, 2010a; Brinkhof *et al.*, 2010b; De Andrés *et al.*, 2005). El uso de técnicas moleculares también ha permitido estudiar la genética del virus y de esta forma obtener información sobre su filogenia, evolución, tropismo y patogenicidad (Crespo *et al.*, 2012; Erhouma *et al.*, 2008; Glaria *et al.*, 2009; Glaria *et al.*, 2012; Leroux *et al.*, 2010; Ramirez *et al.*, 2012; Reina *et al.*, 2006; Shah *et al.*, 2004a), de gran utilidad en estudios epidemiológicos e inmmunopatológicos.

Para la confirmación de la infección tras el diagnóstico clínico y anatomopatológico, se pueden realizar técnicas inmunohistoquímicas mediante el uso de anticuerpos monoclonales y policlonales disponibles comercialmente. Estas técnicas permiten detectar la presencia de antígenos víricos en diferentes tejidos fijados del animal (formol al 10%, líquido de Bouin o sales de zinc) y embebidos en parafina (Benavides et al., 2006b; Gelmetti et al., 2000), permitiendo de esta forma relacionar la distribución y localización del VVM con la lesión y arquitectura del tejido. Se ha detectado antígeno vírico en pulmón (Begara et al., 1995; Benavides et al., 2006b; Gelmetti et al., 2000), SNC (Benavides et al., 2006a; Benavides et al., 2009; Benavides et al., 2006c; Torsteinsdottir et al., 1992), glándula mamaria (Bolea et al., 2006), articulaciones y órganos linfoides (Preziuso et al., 2003a), riñón (Angelopoulou et al., 2006), hígado y corazón (Brellou et al., 2007) y tercer párpado (Capucchio et al., 2003). El anticuerpo anti-p27 del núcleo viral, se utilizó con éxito para estas técnicas, demostrando una gran sensibilidad y especificidad (Gelmetti et al., 2000), sin embargo, no era un anticuerpo comercial y el hibridoma utilizado ya no está disponible para su obtención. Por ello, este anticuerpo no se encuentra disponible actualmente. Existen anticuerpos comerciales pero presentan sensibilidades muy bajas, que dependen en gran medida del genotipo de la cepa infectante.

7. PREVENCIÓN Y CONTROL

7.1. Factores de riesgo

Tal y como se ha sido señalado previamente, las dos fuentes más importantes de contagio de VVM entre animales son la vía horizontal, por contacto con ovejas infectadas, y la lactación natural, a través de calostro o leche (Berriatua *et al.*, 2003a; Blacklaws *et al.*, 2004; Leginagoikoa *et al.*, 2010; Peterhans *et al.*, 2004).

La transmisión horizontal se puede dar tanto por contacto oral como respiratorio, y parece que el contacto entre el cordero y la madre infectada es la forma más eficiente de contagio (Berriatua *et al.*, 2003a; Houwers *et al.*, 1989; Leginagoikoa *et al.*, 2006b; Torsteinsdottir *et al.*, 2003; Álvarez *et al.*, 2005). Se ha relacionado directamente el numero de días que las ovejas permanecen estabuladas, el tamaño del rebaño y el contacto entre sí de los animales, con el incremento de los niveles de infección del rebaño (Blacklaws *et al.*, 2004; Pérez *et al.*, 2010; Simard and Morley, 1991), y se ha establecido que la estabulación permanente de estos animales, el manejo y espacio disponibles son factores a tener en cuanta para el control de la transmisión horizontal de la infección (Leginagoikoa *et al.*, 2006a; Pérez *et al.*, 2010). Aún no se conoce con exactitud el modo de transmisión horizontal del virus, aunque parece que únicamente se transmite a distancias cortas entre animales, por contacto directo, por aerosoles (Leginagoikoa *et al.*, 2010; Villoria *et al.*, 2013) o probablemente por fómites (Gudnadottir and Palsson, 1965; Villoria *et al.*, 2013), aspectos a tener en cuenta para el control de la enfermedad.

La mayor o menor contribución de la vía lactogénica en condiciones naturales en la diseminación de la infección ha sido ampliamente discutido (Broughton-Neiswanger *et al.*, 2010; Houwers *et al.*, 1983; Houwers *et al.*, 1989; Leginagoikoa *et al.*, 2006b; Lerondelle *et al.*, 1995; Preziuso *et al.*, 2004a; Álvarez *et al.*, 2005). Se ha demostrado que existe una correlación positiva entre las posibilidades de seroconversión de un cordero que proviene de madre seropositiva, pero ese riesgo de seroconversión es similar para corderos alimentados con calostro/leche de madres seropositivas y seronegativas independientemente del manejo en el encalostramiento/amamantamiento (Leginagoikoa *et al.*, 2006b). Igualmente, se han asociado positivamente los niveles de seroconversión con el tiempo de vida que pasa el cordero con la madre infectada

seropositiva, en esta situación, el contagio horizontal tiene mayor importancia que el que se establece a través de calostro (Berriatua *et al.*, 2003a).

En este sentido la transmisión vertical parece presentar menor importancia (Blacklaws *et al.*, 2004; Broughton-Neiswanger *et al.*, 2010; Cortez-Romero *et al.*, 2011; Romero *et al.*, 2010; Souza *et al.*, 2013), especialmente en etapas iniciales de los programas de control donde se persigue principalmente reducir los niveles de prevalencia. Sin embargo sí será de gran interés en etapas finales donde el objetivo sea alcanzar la erradicación de la enfermedad del rebaño.

Por otro lado, en el brote islandés se evidenciaron notables diferencias de susceptibilidad entre las razas locales islandesas y las foráneas, siendo las primeras mucho más resistentes a la enfermedad (Cutlip *et al.*, 1986), por lo que el papel del hospedador parece jugar un papel importante en la restricción de la infección y el desarrollo de la enfermedad. Tratando de confirmar esta hipótesis se realizó un estudio reciente en diversas razas para comprobar como influiría el hospedador en la restricción de la cantidad de provirus en sangre periférica, y en dicho estudio se comprobó que el gen Ovar-DRB1 del hospedador contribuye, entre otros genes, a alcanzar ese control y por tanto a limitar la infección (Herrmann-Hoesing *et al.*, 2008). Asimismo, en un estudio estadístico donde se valoraron factores de transmisión de la enfermedad previos al destete, se obtuvo como resultado que las corderas nacidas de hembras seronegativas mayores de 4 años seroconvertían un 50% menos que aquellas nacidas de animales seronegativos o seropositivos menores de esa edad, concluyendo que podría existir un componente de resistencia genética heredable (Berriatua *et al.*, 2003a).

En nuestra comunidad hemos observado que los ovinos predominantemente infectados en nuestra región son de raza Assaf. Este hecho sugiere que la raza Assaf española podría ser más susceptibles a la enfermedad que otras razas autóctonas como la raza Churra (Benavides *et al.*, 2007).

7.2. Programas de control

Actualmente, no existen vacunas disponibles en el mercado para la vacunación de los animales. Se han probado vacunas con el virus completo atenuado o vacunas recombinantes, con plásmidos o proteínas víricas, pero por ahora ninguna ha resultado eficaz en la protección frente al virus (Pepín, 1998; Torsteindottir, 2007). Tras la infección vía intratraqueal con un VVM atenuado y el posterior desafío, se ha observado que aunque la vacuna no produce total protección frente a la infección, si induce una protección parcial frente a la misma, aislándose hasta 10 veces más el virus en animales no vacunados que en animales protegidos por la vacuna (Petursson, 2005). Recientemente se ha probado en ratón una vacuna ADN cuyo plásmido codifica la proteína inmunogénica p25 del VVM que parece obtener una buena respuesta humoral con un alto título de anticuerpos (Henriques, 2009). Asimismo, se están realizando pruebas con diferentes adyuvantes para la estimulación de la respuesta inmune y una mejor respuesta a la vacuna (De Andrés, 2009). Sin embargo, estas vacunas aún no han sido aplicadas en condiciones de campo y su elaboración supone elevados costes, por lo que económicamente no resaltan una alternativa viable en la lucha frente a la enfermedad.

Por lo tanto, los programas de control de la enfermedad han sido la única herramienta disponible para el control de la misma, no disponiéndose de otras alternativas. Así, durante los brotes islandeses de enfermedad de VM, se estableció un estricto programa de erradicación de la enfermedad basado en el sacrificio de los rebaños infectados y el reemplazo de los mismos con animales procedentes de rebaños libres de infección tras un tiempo prudente de vacío sanitario (Houwers *et al.*, 1984; Petursson, 1994). Otra estrategia de control utilizada durante la epidemia islandesa estaba basada en la división de los rebaños en dos grupos, uno compuesto por el rebaño adulto, y el otro compuesto por el grupo de reposición cuyos corderos están retirados inmediatamente después del parto (Petursson, 1994).

Otros programas de control menos drásticos, han sido puestos en práctica en otros países (Holanda, Francia, Italia, Alemania, etc.) principalmente basados en diferentes medidas combinadas (Luján *et al.*, 2001):

1- Sacrificio de animales seropositivos tras la realización periódica de estudios serológicos (Cutlip and Lehmkuhl, 1986).

2- Si el porcentaje de prevalencia y la tasa de reposición lo permiten, sacrificio de la progenie de madres seropositivas, inmediatamente al nacimiento, por las altas probabilidades de infección madre-cordero (Houwers *et al.*, 1989).

3- Si la medida anterior no fuera posible, y las instalaciones y manejo lo permiten, una alternativa es la formación de dos rebaños uno seropositivo y otro seronegativo (Luján *et al.*, 2001).

4- Separación de corderos al nacimiento, y alimentación de la progenie a base de calostro procedente de madres seronegativas o calostro bovino (Houwers *et al.*, 1984; Houwers *et al.*, 1989).

En el valle del Ebro, en España, se ha ideado un método de control aplicado con éxito en rebaños de prevalencias moderadas (alrededor del 50%) que consiste en un único muestreo serológico de todo el rebaño y el marcado indeleble de las ovejas seropositivas. Así se utilizarán como reposición los animales seronegativos e hijas de seronegativas. Esta medida irá acompañada de una vigilancia de cualquier signo clínico característico de la enfermedad que muestren los animales que conducirá a su sacrificio precoz (Ferrer, 1995; Luján *et al.*, 2001).

TRABAJO PREVIO: DESARROLLO DE TÉCNICAS DE INMUNOHISTOQUÍMICA

TRABAJO PREVIO: DESARROLLO DE TÉCNICAS DE INMUNOHISTOQUÍMICA

Para la realización de los dos primeros trabajos de tesis se desarrollaron técnicas de inmunofenotipado celular en tejido incluido en parafina que hasta entonces, no habían sido utilizadas en ovino. En concreto, el inmunofenotipo se determinó en las siguientes poblaciones celulares:

- Linfocitos T: CD3 (panlinfocitos T)
    - Subpoblación CD4+: (17D1, VMRD)
    - Subpoblación CD8+: (AB4055, Abcam)
    - Linfocitos T γδ
- Linfocitos B: CD79αcy
- Macrófagos: CD68

Para la realización de las técnicas de inmunohistoquímica se siguió el siguiente protocolo:

1. Recogida y fijación de las muestras: Las muestras para la fijación fueron recogidas en recipientes de plástico y talladas en fresco con un grosor no mayor de 0.5 cm. Se recogieron las muestras con premura ya que la mayoría de los antígenos que se pretendía detectar eran antígenos que se localizan en la membrana plasmática celular y resultan muy lábiles. Para la fijación de las muestras se utilizó un fijador de sales de zinc, compuesto por 0.5% cloruro de zinc y 0.5% acetato de zinc en un buffer 0.1M Tris base 0.05% de acetato cálcico a pH 7.4. Durante el tiempo de la necropsia se mantuvieron tanto muestras como líquido fijador a temperatura ambiente, para luego conservarlos a 4º C durante 24-48 horas.

2. Inclusión: Las muestras fueron introducidas en un inclusor donde fueron deshidratadas en un ciclo rutinario de inclusión histológica mediante pases por alcoholes de grado creciente, xilol e inclusión en parafina a 60º C.

3. Corte de las muestras: Mediante el uso de un microtomo se realizan secciones de 3 μm que se colocan en portaobjetos previamente tratados con un homopolímero (*Poli-L-lysine, Sigma-Aldrich*) que evita la separación de la muestra del portaobjetos durante la técnica de inmunohistoquímica.

4. Técnica de inmunohistoquímica: La técnica de inmunohistoquímica fue común para la detección de cada fenotipo celular, variando únicamente el anticuerpo primario utilizado, y el secundario para la detección del mismo. Así, tras la hidratación de las muestras, las secciones tisulares fueron tratadas con peróxido de hidrógeno al 3% en agua destilada, durante 30 minutos para bloquear la actividad posterior de la peroxidasa endógena. Posteriormente fueron incubadas durante la noche a 4º C en cámara húmeda con el anticuerpo primario correspondiente. Al día siguiente, las reacciones se desarrollaron con el polímero marcado con peroxidasa EnVision anti-ratón o anti-conejo en función del anticuerpo primario (*EnVision+ System- HRP Labelled Polymer Anti-ratón, Dako, EnVision+ System- HRP Labelled Polymer Anti-conejo, Dako*) por 30 minutos a temperatura ambiente. Este sistema EnVision es útil para la detección de antígenos presentes en concentraciones bajas. La visualización está basada en la peroxidasa (HRP) empleando DAB (*Vector Lab, Burlingame, California, USA).* Este sistema no contiene biotina, por lo que se reduce significativamente la tinción inespecífica derivada de la actividad endógena de la avidina-biotina. Los lavados entre cada paso se realizaron con buffer TBS pH: 7,4 durante 5 minutos (3 repeticiones).

La especificidad de la técnica la evaluamos mediante controles negativos (omitimos la incubación con el anticuerpo primario) y controles positivos (secciones de nódulo linfático).

ANTICUERPOS PRIMARIOS UTILIZADOS:	**DILUCIÓN**
Linfocitos T: CD3 (A0452, DAKO)	1/500
Subpoblación CD4+: (17D1, VMRD)	1/100
Subpoblación CD8+: (AB4055, Abcam)	1/50
Linfocitos B: CD79αcy (HM57, DAKO)	1/20
Macrófagos: CD68 (KP1, DAKO)	1/100
Linfocitos T γδ: (CC15, Serotec)	1/100

Tabla 1. Anticuerpos primarios utilizados en las pruebas de inmunohistoquímica.

PRIMER TRABAJO

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PATTERNS OF LESION AND LOCAL CELL-IMMUNE RESPONSE RELATED TO THE HOST IN NATURAL CASES OF OVINE VISNA. *J Comp Pathol*, Vol. 147, 2011 Elsevier Ltd, England, pp. 1-10.

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ABSTRACT

In this work a detailed description of the nervous form of ovine Maedi-Visna has been carried out by histological and immunohistochemical techniques. The objective of this work was to study the lesion types and the local cell-immune response related to each lesion type, and the possible relationship between them and the host. Thirty-four Assaf ewes were studied, twenty-nine of which had shown nervous signs. After histopathological examination, lesion patterns were described according to: location, extent, and predominance of each cell type. Using immunohistochemical labelling against T cells (CD3+, CD4+, CD8+, γδ), B cells and macrophages, clear differences were identified between cell types in each lesion pattern. Two main lesion types were described: *"Lymphocytic type"*, where areas of mild-moderate injury characterized by a clear predominance of T cells were observed, and "*histiocytic type"*, characterized by more severe lesions with extensive areas of malacia and a large number of macrophages and B cells. Each animal showed a unique lesion pattern and these differences could be due to the individual resistance to the progression of infection. The lymphocytic lesions appear to be initial or latent phases of slow progression, in which the animal presents some sort of natural resistance to the infection. Furthermore, the hystiocytic pattern is more severe and extensive, and may be due to an individual poor immune response or a greater virulence of the viral strain. This work could result useful in future studies based on the individual immune response, in order to control the proggresion of the infection or the disease, in terms of evaluation of vaccines, treatments or individual resistances.

Keywords: Maedi; Visna; sheep; lentivirus; immunopathology.

INTRODUCTION

Maedi-Visna (MV) is a slow, progressive multi-systemic sheep disease caused by the Maedi-Visna virus (MVV). MVV is a retrovirus of the genus *Lentivirus* that belongs to the small-ruminant lentivirus (SRLV) group and is worldwide distributed. SRLV group includes all the strains of MV and Caprine Arthritis Encephalitis virus (CAEV) (Zanoni, 1998). The disease commonly affects adult sheep and leads to a multi-systemic syndrome characterized by progressive weight loss and chronic non-purulent lung, mammary gland, joint and central nervous system (CNS) inflammation (Cutlip *et al.*, 1988; Dawson, 1987).

In Spain, it is considered a widespread disease, although the data vary according to different studies (Alba *et al.*, 2008; Benavides *et al.*, 2006c; Leginagoikoa *et al.*, 2006b; Lujan *et al.*, 1991; Luján *et al.*, 1993). SRLV seroprevalence in the north of Spain has been estimated at 77%, but on half the flocks of the Spanish Assaf breed, the value overshot 80% (Leginagoikoa *et al.*, 2006b). These flocks are under a specific management whereby dairy sheep are kept in an intensive indoor farming set-up. Several Spanish Assaf flocks reached a high seroprevalence in our region, estimated at 96.8% by an epidemiological study (Sotelo, 1998).

The disease most commonly presents itself in respiratory and mammary forms, while the nervous form has often been sporadic (Benavides *et al.*, 2006a; Benavides *et al.*, 2009; Lujan *et al.*, 1991; Sigurdsson *et al.*, 1957). Recently, numerous cases of the nervous form (even in animals 4-6 months of age) have been diagnosed, especially in the Assaf breed (Benavides *et al.*, 2007; Leginagoikoa *et al.*, 2010). Nervous clinical signs often include hind leg weakness, pelvic limb ataxia, hypermetria or paralysis usually leading to prostration, although the animal remains alert and responds to external stimuli (Benavides *et al.*, 2009; Benavides *et al.*, 2006c; Christodoulopoulos, 2006; Sigurdsson *et al.*, 1957). Lesions in the brain or spinal cord are usually not macroscopically apparent, but occasionally, severe brain injuries are identified as malacia liquefaction areas in the white matter of the encephalon and wedge-shaped areas in the spinal cord (Benavides *et al.*, 2006a; Benavides *et al.*, 2009; Sigurdsson *et al.*, 1957; Sigurdsson and Palsson, 1958). As regards to histological lesions in the central nervous system, chronic non-purulent meningoencephalomyelitis, usually accompanied by demyelination, is a common finding. Mononuclear infiltration in the

choroid plexus that even results in the development of lymphoid follicles was also observable (Cutlip *et al.*, 1979). Histological lesions have been classified into three main patterns in a recent publication (Benavides *et al.*, 2009), according to the location of the lesion and regardless of the cell population comprising the lesion: *Vascular*: mononuclear cells are arranged around blood vessels forming perivascular cuffs; *infiltrative*: additionally a non-purulent infiltrate appears at the neuroparenchyma; and *malacic*: being demyelination the main feature. In this study, the presence of more than one of these patterns in individual affected animals may be seen.

The importance of cell-mediated inmunity in relation to the severity of the nervous lesions has been demostrated in previous studies of sheep intracerebrally infected with MVV (Torsteinsdottir *et al*, 1992). In this sense, the determination of the different cell phenotypes present in the nervous lesions of natural cases of MV has not been previously done to the author´s knowledge; however other studies based on different types of immunological cells exist in its respiratory or mammary forms (Christodoulopoulos, 2006; Cordier *et al.*, 1992; Lujan *et al.*, 1991).

Hence, the observation of a lesion pattern in each animal could be associated with individual differences in the local immune responses. In this way, the aim of the present study was the observation of specific lesion patterns in each individual, which in turn could be associated with differences in the local cell immune response.

MATERIALS AND METHODS

Animals

The necropsy of 34 sheep submitted to the Pathology Diagnosis Service of the Veterinary School of León was performed. The animals came from different flocks (of the Spanish Assaf breed) subjected to the intensive indoor farming system widespread

in the northwest of Spain. Twenty-two animals were adults older than 2 years, nine sheep were between 1 and 2 years and 3 of them were between 4 and 6 months old.

Histopathology

Tissue samples from different organs were taken for histological examination, with special attention to the lung, mammary gland, joints and CNS (CNS samples were obtained systematically, taking different sections: cortex, diencephalon, corpus callosum, hippocampus, midbrain, cerebellar cortex, pons and cerebellar peduncles, medulla oblongata, and cervical, thoracic and lumbar spinal cord). Sample tissues were fixed in 10% buffered formalin for 24 hours at room temperature. Sections of 3 µm thickness of formalin fixed tissue were stained with hematoxylin-eosin.

Immunohistochemistry

In order to characterized the cell phenotypes by immunohistochemistry, nervous sample tissues from 16 sheep, were briefly inmersed in zinc fixative salts (ZSF), 0.5% zinc chloride, 0.5% zinc acetate in 0.05% Tris buffer 0.1M calcium acetate, pH = 7.4, (González *et al.,* 2001) for 24 h at 4º C. After fixation, samples were dehydrated in alcohols of decreasing grades before being embedded in paraffin. Nerve tissue sections of 3 µm thickness from 16 animals were labeled by immunohistochemistry to characterize cell populations in different patterns, using a polymer-based detection system (EnVision + System Labeled Polymer-HRP Anti-mouse, Dako, EnVision + System Labeled Polymer-HRP Anti-rabbit, Dako). Tissue samples were dewaxed and rehydrated, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 minutes. The nonspecific links were blocked for 20 minutes with 1/20 goat serum diluted in TBS-BSA (TBS: 0.5M Tris Base, 9% NaCl, pH 7.4, TBS-BSA 0.9% bovine serum albumin). Slides were incubated overnight with the primary

antibody against T cells (CD3+, CD4+, CD8+ and γδ), B cells (CD79 αcy) and macrophages (CD68) (Table 1). Subsequently, the sections were incubated for 30 minutes using the EnVision+ system and developed with a solution of 3-3-Diaminobezidine (DAB) (Vector Lab, Burlingame, California, USA). The slides were stained with hematoxylin for 12 seconds and mounted. 5-minute washes with TBS were performed twice between each step. The specificity of the technique was evaluated by negative controls (omitting the incubation with the primary antibody and incubating it with non-immune sera) and positive controls (lymph node sections and positive samples of previously tested as MVV-positive). Results were subjectively scored as: + / -, (a few scattered positive cells), + (moderate number of positive cells), + +, (several number of positive cells) and + + + (clear predominance). Samples of a sheep without characteristic lesions of MV that showed negative results to PCR procedures and serology were also included as negative control.

Formalin-fixed nerve tissue samples from 24 animals were tested by immunohistochemical techniques against the p27 viral antigen (Gelmetti, 2000), as previously described (Benavides *et al.*, 2006b).

Table 1.

List of primary antibodies

Antibody (clone)	Host species	Source
PanT cells CD3+ (A0452)	Rabbit	DAKO
T cells CD4+ (17D1)	Mouse	VMRD
T cells CD8+ (AB4055)	Rabbit	Abcam
B cells CD79 αcy (HM57)	Mouse	DAKO
Macrophages CD68 (KP1)	Mouse	DAKO
T cells γδ (CC15)	Mouse	Serotec

Serological Tests

Serum samples were obtained from all the sheep to evaluate the presence of antibodies against MVV using a commercial test (Elitest®, Hyphen BioMed, Neuville/Oise, France), in accordance with the manufacturer's instructions. ELISA results were reported as positive or negative on the basis of the cut-off value calculated as per manufacturer's instructions (absorbance 450 nm).

PCR Procedures

Genomic DNA, from 10 animals that do not were tested by immunohistochemistry against MVV, was extracted from peripheral blood mononuclear cells and tissue samples using QIAamp® DNA Blood Mini Kit (QIAGEN). Amplification of LTR and *gag* regions from animals with nervous lesions was performed as previously described (Reina *et al.*, 2006).

RESULTS

All the animals examined presented characteristic lesions of MV disease, although clear differences appeared among them.

Histopathology and Immunohistochemistry

The description and characterization of nervous lesions were carried out based on location, extent of injury, and predominant cell type (Fig. 1.) and the results of immunohistochemical studies are summarized in table 2:

Lymphocytic type: Non-purulent encephalitis composed mainly of lymphocytes characterizes this lesion pattern. Scattered vacuoles of demyelination are occasionally seen. This type was subdivided into two lesion subtypes:

Lymphocytic Type A: the lesion is found in the choroid plexus and meninges, showing no apparent lesion or only mild lesions in the adjacent neuroparenchyma. Hyperplasia of the lymphoid tissue, which even develops aggregates or lymphoid follicles, is observed (Fig.1).

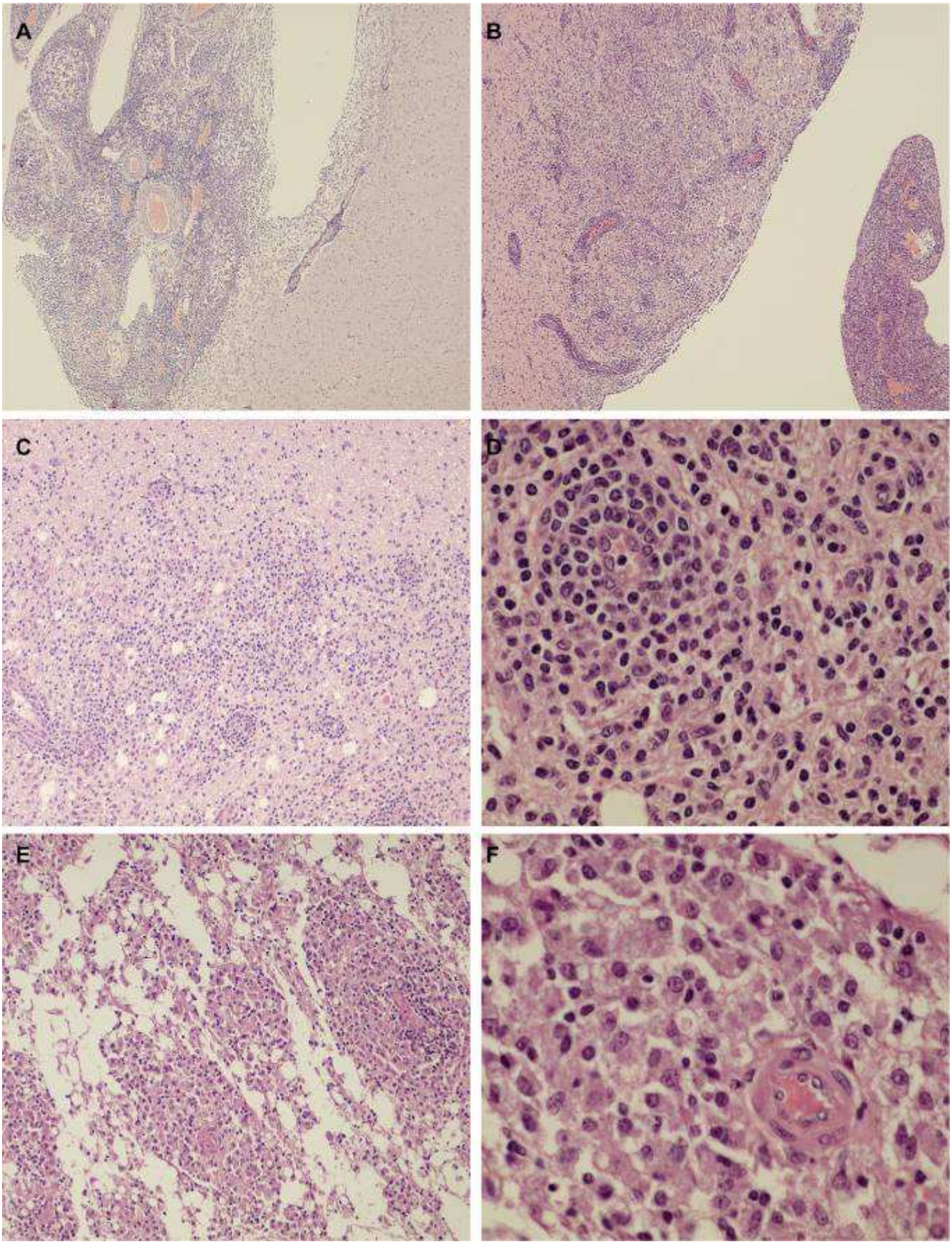

Figure 1

***Fig. 1**. Nervous lesions in natural cases of ovine Visna. Histopathological classification: (A) Lymphocytic type A, a non-purulent choroiditis composed mainly of lymphocytes characterized this pattern, showing no apparent lesion or only mild lesions in the adjacent neuroparenchyma. Hyperplasia of the lymphoid tissue, which even develops aggregates or lymphoid follicles, is observed. (B) Histiocytic type A, the main lesion is located in the choroid plexus and meninges, and the adjacent neuroparenchyma presents moderate-severe damage. A large amount of macrophages at the damaged periventricular area, demyelination vacuoles and perivascular cuffs are commonly observed. (C) Lymphocytic type B, a non-purulent encephalitis characterized by a mononuclear cell infiltrate with a clear predominance of lymphocytes with several macrophages between them, several gliosis foci, as well as the arrangement of round cells around blood vessels forming perivascular cuffs are the most significant changes (D) Higher magnification of image 1.C. (E) Histiocytic type B, the neuroparenchyma is the most affected area. Extensive areas of malacia and a non-purulent infiltrate with evident predominance of large and foamy macrophages ("gitter cells") as compared to lymphocytes are observed. (F) Higher magnification of image 1.E. HE. (A-B) x40, (C) x100, (D) x400, (E) x100, (F) x400.*

In these types of lesion there is a clear predominance of T cells (CD3+) in the infiltrate of the choroid plexus and meninges (Fig. 2). A high proportion of CD4+ versus CD8+ T cells is observed in mild choroiditis and meningitis, while the percentages become similar in lesions with the presence of lymphoid agreggates/follicles. These lymphoid aggregates appear to arrange themselves into a pattern of lymphoid organ follicles with large amounts of CD4+ T cells located in the incipient active germinal center areas, whereas CD8+ lymphocytes can be found both in the periphery and in the center of follicles near the macrophages. B cells are rarely detected, except in the areas of well-developed lymphoid follicles where the germinal center will be composed mainly of B cells. In the case of mild lesions in the adjacent neuroparenchyma, monolayer cells around blood vessels are mainly composed of CD4+ T cells, together with CD8+ lymphocytes in multi-layered perivascular cuffs. CD8+ cells also appear adjacent to the periventricular neuroparenchyma and in small gliosis foci. Few γδ lymphocytes and macrophages are observed through the infiltrate of the choroid plexus and the neuroparenchyma.

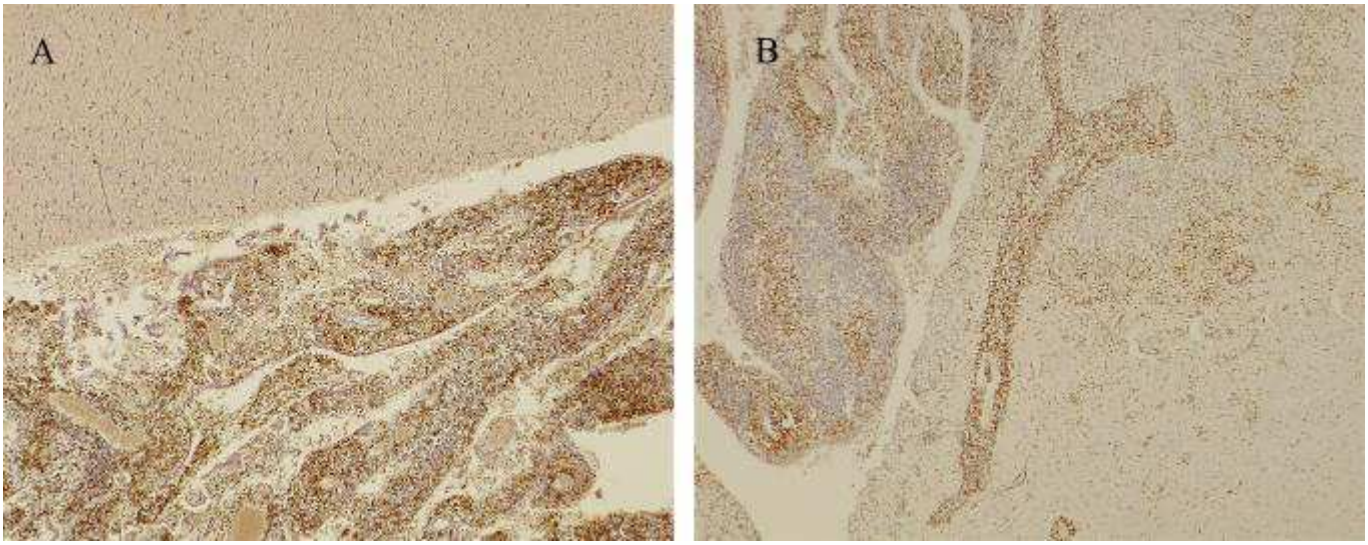

Fig. 2 *Phenotypic characterization of cell populations present in each histological type: (A) Lymphocytic type A, there is a clear predominance of T cells in the infiltrate of the choroid plexus and meninges. Occasional T lymphocytes located in the adjacent neuroparenchyma. (B) Histiocytic type A, small number ot T cells in choroid plexus compared with lymphocytic type A. Numerous T cells in the adjacent damaged neuroparenchyma. Anti-CD3+ labelling. IHC. x40.*

Lymphocytic Type B: in this case, the main lesion is located in the neuroparenchyma, with the choroid plexus and meninges also appearing slightly infiltrated, mainly by lymphocytes. A mononuclear cell infiltrate with a clear predominance of lymphocytes with several macrophages between them, several gliosis foci, as well as the arrangement of round cells around blood vessels forming perivascular cuffs are the most significant changes (Fig. 1). Likewise, in this pattern T cells (CD3+) are also the most abundant cells. Both the meninges and the choroid plexus are slightly infiltrated by T lymphocytes, mainly by CD8+. An extensive infiltration of T cells, which are located above all at the edges of the lesion, is observed along the neuroparenchyma (Fig. 3). Most of the T cells are CD8+ in gliosis foci and around vessels, together with CD4+ cells in a lower percentage. CD4+ lymphocytes appear in a similar number as CD8+ in multi-layered perivascular cuffs (Fig. 4). Macrophages in a moderate number are mainly located in the neuroparenchyma of the central area of the lesion, or at the edges of the lesion around slightly damaged blood vessels forming mono/bi-layers (Fig. 3). Few γδ and B lymphocytes are detected, with

γδ T cells being scattered along the parenchyma and B cells always close to the vessels (Fig. 5).

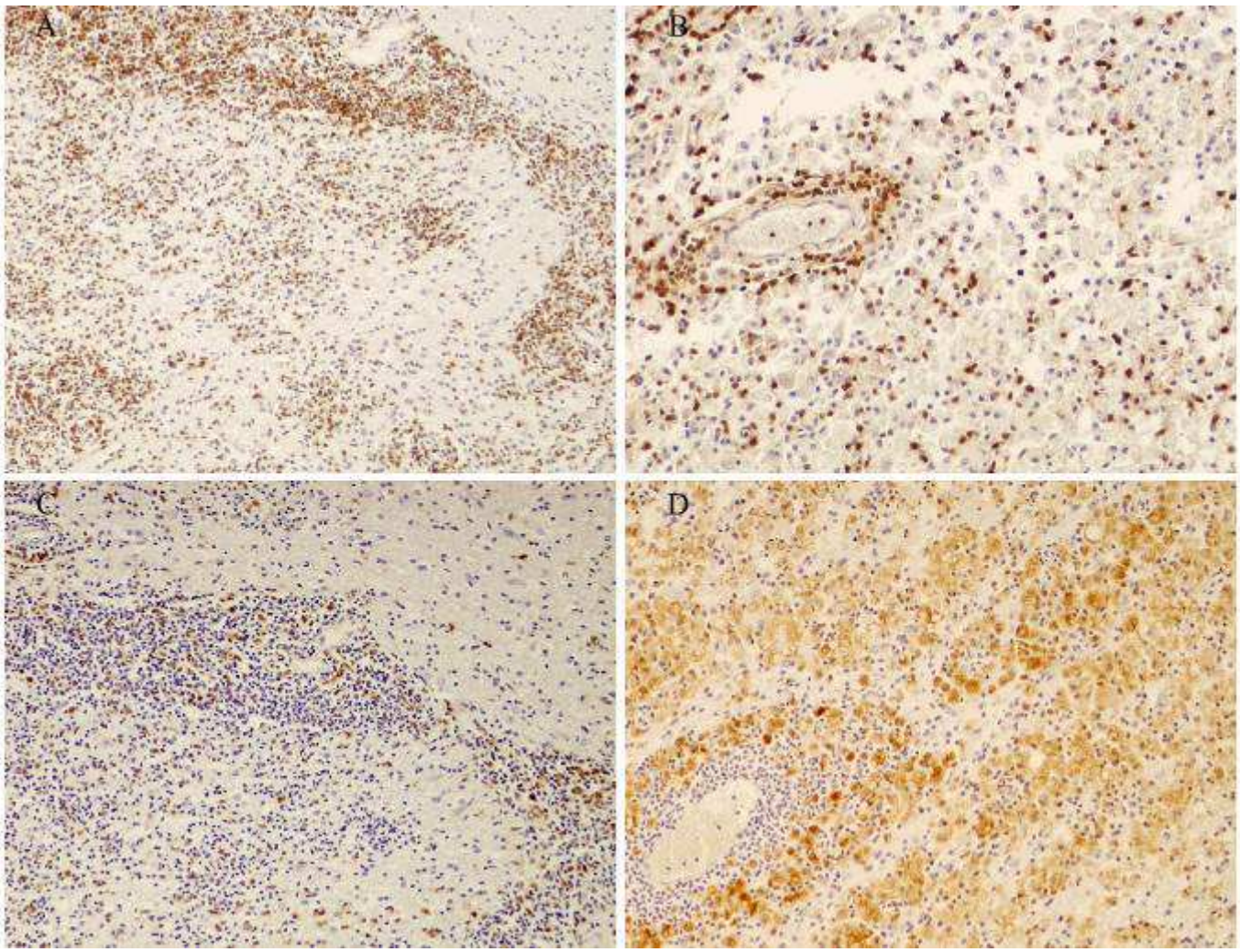

Fig. 3 *Phenotypic characterization of cell populations present in each histological type: (A) Lymphocytic type, anti-CD3+ labelling: an extensive T cells immunolabelling in perivascular cuffs and the surrounding parenchyma. (B) Hystiocytic type, anti-CD3+ labelling: T cells immunolabelling appears in a lower proportion than in lymphocytic type. (C) Lymphocytic type, anti-macrophages labelling: macrophages in a moderate number located in the central area of the lesion and around blood vessels. (D) Hystiocitic type, anti-macrophages labelling: clear predominance of macrophages with foamy cytoplasm along the lesion. IHC. (A) x100, (B) x150, (C) x100, (D) x150.*

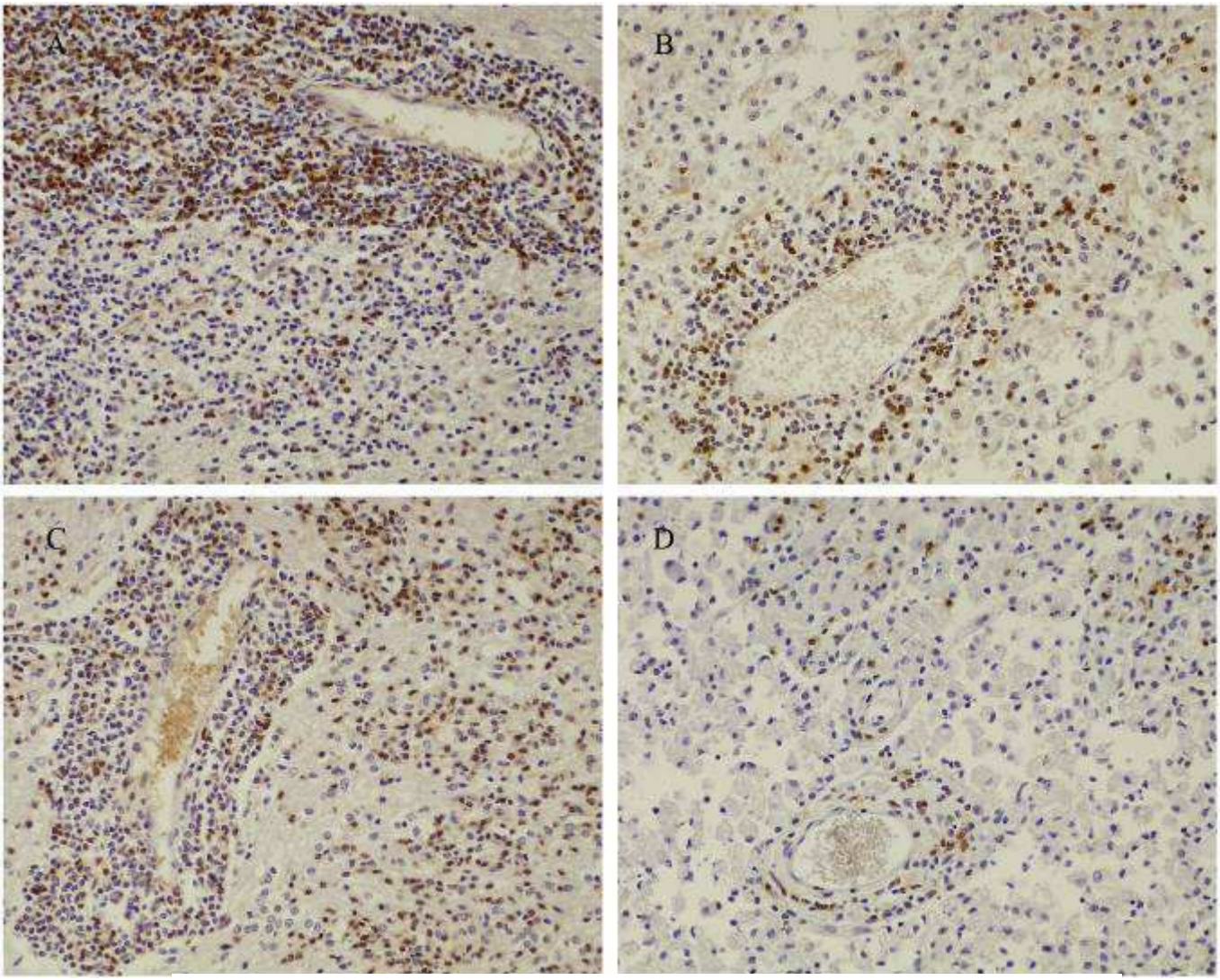

Fig. 4 *Phenotypic characterization of cell populations present in each histological type: (A) Lymphocytic type, anti-CD4+ lymphocytes labelling: CD4+ lymphocytes immunolabelling in multi-layered perivascular cuffs and in a low proportion along the neuroparenchyma. (B) Hystiocytic type, anti-CD4+ lymphocytes labelling: CD4+ lymphocytes located closer to the endothelium. (C) Lymphocytic type, anti-CD8+ lymphocytes labelling: numerous CD8+ T cells in the perivascular cuffs and infiltrating the neuroparenchyma. (D) Hystiocitic type, anti-CD8+ lymphocytes labelling: CD8+ lymphocytes appear in a lower proportion along the lesion than in lymphocytic types. IHC. x200*

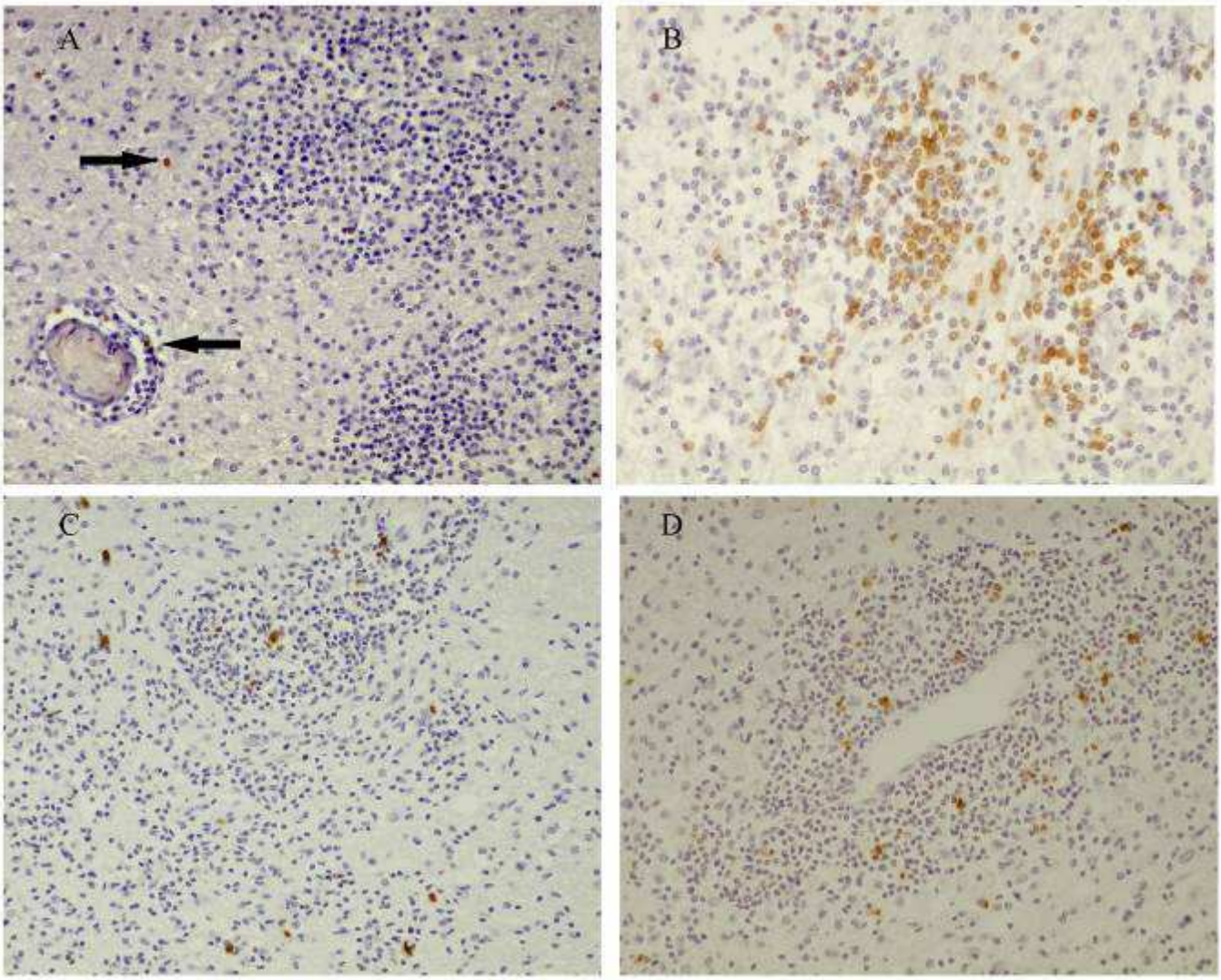

Fig. 5 *Phenotypic characterization of cell populations present in each histological type: (A) Lymphocytic type, anti-B lymphocytes labelling: arrows indicate occasional B cells. (B) Hystiocytic type, anti-B lymphocytes labelling: extensive B cells immunolabelling in the lesion. (C) Lymphocytic type, anti-γδ lymphocytes labelling. (D) Hysticytic type, anti-γδ lymphocytes labelling: Small number of γδ T cells in lymphocytic and hystiocytic types, scattered along the neuroparenchyma and closer to the endothelium. IHC. x200.*

Histiocytic type: This pattern is characterized by severe lesions, where the predominance of macrophages with foamy cytoplasm and extensive areas of malacia are the main pathological changes. As in the previous case, two subtypes were established: ***Histiocytic Type A:*** the main lesion is located in the choroid plexus and meninges, and the adjacent neuroparenchyma presents moderate-severe damage. Although infiltration of the choroid plexus is predominantly lymphocytic, a greater number of macrophages are present in contrast to lymphocytic type A. A clear predominance of macrophages at the damaged periventricular area, demyelination vacuoles and perivascular cuffs are commonly observed (Fig. 1). The choroid plexus infiltrate is composed mainly of T and B cells, but with a high proportion of macrophages between them. Numerous B cells are observed in the lymphoid follicles and along the periventricular parenchyma. Macrophages with expanded foamy cytoplasm are clearly the predominant cells in the adjacent damaged neuroparenchyma, where they appear together with a lower proportion of T cells (Fig. 2). The T cells observed are mostly CD8+ T cells located in gliosis foci and in perivascular cuffs. CD4+ lymphocytes appear in a lower proportion, mainly located around the blood vessels. Many macrophages and lymphocytes are detected around multi-layered perivascular cuffs, while in vessels with mild damages at the edge of the lesion only a single layer of macrophages adjacent to the endothelium was observable. There are also plenty of B cells, mainly located next to the vessels in the periphery of the lesion. In this case, γδ cells are also scattered among the other cell types along the area of the lesion.

In ***Histiocytic Type B***, the neuroparenchyma is the most affected area, and the most severe lesions appear in this pattern. A non-purulent infiltrate with evident predominance of large macrophages as compared to lymphocytes is observed. Clusters of “gitter” cells are frequently observed near blood vessels, forming large cuffs. This is usually accompanied by intense vacuolation foci that coalesce, forming extensive malacic areas (Fig. 1). In this type of lesion, the clear predominance of macrophages with foamy cytoplasm and demyelination in the neuroparenchyma are the main features (Fig. 3). T cells are mainly ranged along the edges of the lesion (Fig. 3). In this pattern, the proportion of CD8+ is also higher than CD4+. Perivascular cuffs are composed of several layers, with the external ones mainly consisting of macrophages behind a barrier of CD8+ and CD4+ lymphocytes located closer to the endothelium (Fig. 4). Slightly damaged vessels are completely surrounded by macrophages. In this type, a

considerable number of B lymphocytes were found close to endothelium, especially at the edges of the lesion, and some γδ cells were detectable along the lesion (Fig. 5).

The 24 sheep (4 showed lymphocytic type B lesion, 7 hystiocytic type A lesion and 13 hystiocytic type B lesion) tested by immunohistochemistry against MVV showed positive labelling of p27 MVV antigen. This positive signal was mainly characterized by fine granular deposits in the cytoplasm of macrophages-like cells. Although the assessment was done subjectively, positive staining was clearly more abundant in the histiocytic type lesions than in the lymphocytic ones.

Serological test and PCR procedures

10 animals tested by PCR methods were positive and the 34 animals yielded positive results to the serological tests.

Table 2.

Immunohistochemistry results.

Cell types	Lymphocytic A	Lymphocytic B	Histiocytic A	Histiocytic B
T cells	++	+++	++	++
Macrophages	+	+	+++	+++
CD8+ T cells	+	+++	++	++
CD4+ T cells	++	++	+	+
B cells	+/-	+/-	++	++
γδ T cells	+	+/-	+/-	+/-

Table 2: Immunohistochemistry results were subjectively scored as: + / -, (a few scattered positive cells), + (moderate number of positive cells), + +, (several number of positive cells) and + + + (clear predominance).

DISCUSION

The nervous form of MV has been previously studied by several authors who have described the lesions associated with the disease (Benavides *et al.*, 2006a; Benavides *et al.*, 2009; Sigurdsson *et al.*, 1957; Sigurdsson and Palsson, 1958). These studies have been taken as a reference for the histopathological study and diagnosis of the disease. The determination by immunohistochemistry of the cell population of each lesion type is of interest in understanding the pathogenesis of the disease, due to the important role of cell-mediated immunity in relation to the severity of the nervous lesions (Torsteinsdottir *et al.*, 1992). Likewise, the balance between cell populations and their interactions seem to determine the evolution of viral infection, (Dorries, 2001), as this may occur in our cases, given that the significant differences found in this study between the lesions of the animals affected included in this study. Thus, based on these observations one lesion pattern can be established in each animal affected.

In this sense, an important feature was the finding in this study of the lymphocytic type, with lesions located almost exclusively in the choroid plexus and meninges with mild lesions in the adjacent neuroparenchyma *(lymphocytic type A)* that showed a clear predominance of T cells, particularly CD4+. The description of this lesion type is very similar to previously described lesions in early stages of CNS infection by other lentivirus (CAEV, FIV and HIV-1), where up to 70% of lymphocytes and 10% of macrophages/microglia are observed around the perivascular cuffs. Also, CD4+ cells are more numerous in perivascular sleeves while the CD8+ infiltrate is mainly distributed in the neuroparenchyma (Torsteinsdottir *et al.*, 1992). Thus, this lesion type could be related to early stages of the disease or different mechanism of resistance to the progression of the infection. In addition, neuroinvasion of MVV may be initiated by activated peripheral T cells that are obliged to previously migrate to the CNS to facilitate monocyte recruitment (Cheblounе *et al.*, 1998). Moreover, an experimental study showed that immunosupression with antilymphoid serum prevents cell proliferative response and the development of lesions (Panitch *et al.*, 1976). Due to these observations, the presence of lymphocytes in mild lesions and in layers around vessels (lymphocytic types) suggests an initial arrival of lymphocytes at the CNS and might represent the first response to viral infection. Besides, initial mammary and arthritic (CAE) lesions have also been studied in infected experimental animals,

showing an accumulation of activated CD4+ and CD8+ cells in tissues, mainly around blood vessels with only a few B as in the lymphocytic types (Cordier *et al.*, 1992; von Bodungen *et al.*, 1998).

In our work, *lymphocytic types B* showed severe lesions, with the number of CD8+ cells in the infiltrate of the neuroparenchyma significantly higher than in other patterns. Although CD8+ cells are thought to be important in controlling viral replication, its role in the CNS is still unknown. Indeed, increased tissue damage due to the cytotoxic effect or to an exacerbated pro-inflammatory cytokine secretion has also been suggested (Torsteinsdottir *et al.*, 2007). It is known that following viral infection of the CNS, a Th-1 type immune response stimulates the CD8+ T lymphocyte migration, which results in a tremendous up-regulation and secretion of chemotactic substances, and consequently, an increasing number of inflammatory cell are attracted to the site of virus infections (Dorries, 2001). In this sense, this lesion type could be representing a transitional step toward the more severe lesions, or another mechanism of immune response wherein the importance of CD8+ cells seems to be very significant

On the other hand, both *histiocytic type A* and *histiocytic type B* showed severe lesions in the neuroparenchyma and could be representing an advanced stage with the recruitment of monocytes/macrophages due to an exaggerated but probably ineffective immune response. In this regard, in previous observations of lentiviral infections (SIV, HIV-1) studying later stages of the disease, a monocyte/macrophage arrival to form 90-95% of the cellular infiltrate has been described, with almost all of the rest of the cell population being CD8+ cells (Kim *et al.*, 2004; Lackner *et al.*, 1991). Moreover, CAE arthritic lesions of both naturally and experimentally infected animals with advanced lesions present a greater accumulation of macrophages and B cells in the inflammatory tissue (von Bodungen *et al.*, 1998). Thus, the increase in B cells suggests that the immune response to infection may have tended towards a Th2 type of immune response, losing effectivity, as has been previously described in arthritic lesions of goats infected with CAEV (Wilkerson *et al.*, 1995). Likewise, evidence for an ineffective T cell response have been described , *in vitro,* in clinically severely affected animals in terms of antigen recall responses due to impaired presentation defficient in costimulatory molecules (Reina *et al.*, 2007). According to this, all the sheep affected in less than a year (seven sheep) presented the histiocytic type of lesions, thus, this

suggests that these lesions may have a relatively rapid development, and consequently represent an ineffective immune response.

In our cases, it seems that the amount of antigen is much large in relation to the hystiocytic types; this could be due to the large number of macrophages located in this type of lesions, or to a lesion pattern with low resistance to viral replication. However IHQ against MVV has not been carried out on enough number of animals to consider this feature as significant.

Differences in the location of the main choroid, plexus/meninges (type A), or neuroparenchyma (type B) lesion could also be explained by the viral route of entry to the CNS, by the blood–brain barrier (BBB) or by the blood–cerebrospinal fluid barrier. Viral migration through the BBB to reach the neuroparenchyma could be the first step in developing encephalitis (type B), while in the case of migration through the choroidal/meningeal endothelial barrier, choroiditis/meningitis would be the expected lesions (type A). Both MVV routes of entry to the CNS have been proposed by means of different mechanisms of migration (Benavides *et al.*, 2009; Broadwell, 1993; Drevets and Leenen, 2000; Georgsson, 1994; Georgsson *et al.*, 1989; Haase *et al.*, 1977; Peluso *et al.*, 1985).

The pathogenesis of the disease has been hypothesized by other authors: the damage of the BBB due to the entry of MVV will result in a release of several pro-inflammatory cytokines such as TNF-α and the over-expression of endothelial adhesion molecules, leading to changes in vascular permeability and the increased migration of inflammatory cells to the CNS (Craig *et al.*, 1997). This step could be represented by the lymphocytic types of our classification. Then, a continuous immune activation will induce the differentiation and attraction of B cells and macrophages on the tissue level and an increase in the secretion of cytokines, thereby creating a vicious circle (Ebrahimi *et al.*, 2000; Torsteinsdottir *et al.*, 2007). According to this, histiocytic types seem to be advanced stages with severe consequences due to the numerous observed macrophages and B cells. Numerous viral strategies to escape immune-mediated cellular responses have been described; resulting in a life-long persistence of the virus in the brain, thus the presence of long-term progressor animals could be due to this control of viral replication and could be represented by the described lymphocytic types.

In conclusion, these lesion types could be related to different stages or different mechanisms of resistance to the disease. The lymphocytic lesions appear to be initial or latent phases of slow progression, in which the animal presents some sort of natural resistance to the infection. Furthermore, the hystiocytic pattern is more severe and extensive, and may be due to an individual poor immune response or a greater virulence of the viral strain. This detailed description of nervous lesions would represent an improvement in the study of individual response to MVV infection, and the evolution of the disease, and its diagnosis. Likewise, the present work could turn out useful in future studies of this pathology based on the individual immune response to control the progression of the infection, such as evaluation of vaccines, treatments or individual resistance to the disease.

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SEGUNDO TRABAJO

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PERIVASCULAR INFLAMMATORY CELLS IN OVINE VISNA/MAEDI ENCEPHALITIS AND THEIR POSSIBLE ROLE IN VIRUS INFECTION AND LESION PROGRESSION. *Journal of NeuroVirology* 2012, Volume 18, Issue 6, pp 532-537

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A**BSTRACT**

We examined the distribution in the perivascular spaces of Visna/maedi antigen, T-cells (CD3+, CD4+, and CD8+), B-cells and macrophages by immunohistochemistry in 22 natural cases of Visna/maedi encephalitis. Sheep showed *lymphocytic* or *hystiocytic* lesions. In mild lymphocytic lesions the viral antigen was detected in perivascular cuffs where CD8+T-cells predominated, but in severe lymphocytic lesions sparse antigen was identified and CD8+/CD4+T-cells appeared in a similar proportion in multilayer perivascular sleeves. In hystiocytic lesions, vessels were surrounded by macrophages with abundant viral antigen, with CD8+/CD4+T-cells and B-cells in the periphery. These results could reflect different stages of virus neuroinvasion and clarify the neuropathogenesis of Visna/maedi encephalitis.

Keywords: Maedi; Visna; sheep; encephalitis; neuropathology, immunology.

INTRODUCTION

Visna/maedi virus (VMV) is a lentivirus of the Retroviridae family which is related to human immunodeficiency virus (HIV-1) (Thormar, 2005) and causes a slow, progressive multi-systemic disease in sheep. Visna/maedi disease (VM) is mainly characterised by chronic inflammation of the lungs, central nervous system (CNS), mammary glands, and joints (Cutlip *et al*, 1988; Dawson, 1987). The disease is most commonly presented in respiratory and mammary forms, while the neurological form has often been sporadic (Benavides *et al*, 2006a; Benavides *et al*, 2009; Lujan *et al*, 1991; Sigurdsson *et al*, 1957). The main histologic changes are interstitial inflammation of the lungs and mammary glands with proliferation of lymphoid tissue, as well as non-purulent encephalitis and demyelination of the CNS, including the spinal cord (Benavides *et al*, 2009; Georgsson *et al*, 1976; Lujan *et al*, 1991; Sigurdsson *et al*, 1957).

In the the region of Castilla y León (Spain), VM is considered a widespread disease, with a prevalence estimated at 77%, especially in the Assaf dairy flocks subjected to an intensive farming set-up (Leginagoikoa *et al*, 2006). In this region, a previous study showed that a proportion as high as 11. 2% of the sheep showing nervous clinical signs were suffering from the VM (Benavides *et al*, 2009; Benavides *et al*, 2006c; Gómez *et al*, 1999). Nervous clinical signs often include progressive ataxia, limb weakness and paresis, particularly in the hindlimbs, usually leading to total paralysis and recumbency although the animal remains alert(Benavides *et al*, 2006c; Christodoulopoulos, 2006; Polledo *et al*, 2011; Sigurdsson *et al*, 1957). The primary lesion in the brain or spinal cord is a non-suppurative encephalitis predominately periventricular and paraventricular, accompanied or not by non-suppurative choroiditis and meningitis (Benavides *et al*, 2009; Polledo *et al*, 2011; Sigurdsson *et al*, 1957).

The immune response against VMV seems to play a major role in the pathogenetic mechanism, thus an imbalance in the immune response, whether excessive or deficient, would result in lesion development (Blacklaws, 2012; Polledo *et al*, 2011; Torsteinsdottir *et al*, 2007; Torsteinsdottir *et al*, 1992). Once in the host, the main targets of VMV are monocytes/macrophages and dendritic cells, which carry the viral DNA in blood with minimum transcription until the monocytes mature into

macrophages in the tissue of affected organs ("Trojan horse" mechanism)(Peluso *et al*, 1985). It appears that the entry of VMV into the CNS may damage the blood-brain barrier (BBB), leading to changes in vascular permeability and increasing migration of inflammatory cells (lymphocytes and monocytes) to the CNS with secretion of cytokines, creating a vicious circle (Craig *et al*, 1997; Ebrahimi *et al*, 2000; Georgsson, 1994; Georgsson *et al*, 1976; Torsteinsdottir *et al*, 2007). However, this pathological mechanism is not yet fully understood.

A previous study has shown that one sheep with VM nervous lesion showed changes characterized by a diffuse inflammatory infiltrate in the neuroparenchyma with a CD4/CD8 ratio of 0.8, and in the perivascular spaces composed of lymphocytes with a CD4+/CD8+ ratio of 1/3 and 10% monocytes (Torsteinsdottir *et al*, 1992), similar to other lentivirus (CAEV, FIV and HIV-1) (Georgsson, 1994). Recently, two main patterns of lesion have been described in relation to each animal: a *lymphocytic type*, where areas of mild-moderate injury characterised by a clear predominance of T cells, mainly CD8+ lymphocytes, are observed; and a *histiocytic type*, characterised by more severe lesions with clear predominance of macrophages, many of them with foamy cytoplasm, mixed with B cells, that is usually accompanied by intense vacuolation foci that coalesce, forming extensive malacic areas.

These lesion patterns could be related to different stages or mechanisms of resistance to the disease. Thus, the lymphocytic lesions could represent some sort of natural resistance to the infection where initial or latent phases are included, and the hystiocytic pattern may be the result of an individual poor immune response or greater virulence of the viral strain (Polledo *et al*, 2011). In this study we investigated the immunophenotype and distribution of the inflammatory cells specifically within the vascular spaces in relation to the viral antigen and the different patterns of lesion, for the purpose of studying the role of the perivascular spaces in virus neuroinvasion and in the development of the neuroparenchymal lesions (hystiocytic or lymphocytic lesions).

Brain tissue samples from natural cases of Visna/maedi encephalitis in 22 adult sheep (over 2 years-old) of the Spanish Assaf breed were examined. All of them were naturally infected cases which had been submitted to the Pathology Diagnosis Service of the Veterinary School of León with nervous clinical signs, and had been diagnosed as

VM infected. CNS tissue samples were obtained systematically, taking different sections: cortex, diencephalon, corpus callosum, hippocampus, midbrain, cerebellar cortex, pons and cerebellar peduncles, medulla oblongata, and cervical, thoracic and lumbar spinal cord. Sample tissues were fixed in 10% buffered formalin, stained with hematoxylin-eosin (HE) and examined by light microscopy. Samples from these same locations were fixed in zinc salt fixative (0.5% zinc chloride, 0.5% zinc acetate in 0.05% Tris buffer, 0.1M calcium acetate, pH = 7.4), and tested using immunohistochemical techniques (IHC) using serial tissue sections. These serial tissue section were stained with primary antibodies raised against the p27 VM viral antigen (Gelmetti *et al*, 2000), as previously described (Benavides *et al*, 2006b), and T cells (CD3+, CD4+, and CD8+), B cells (CD79 αcy) and macrophages (CD68), using previously described IHC staining procedures (Polledo *et al*, 2011). Samples from a sheep without characteristic lesions of VM which showed negative results to PCR procedures and serology were used as negative control. A semiquantitative analysis of the presence of the different cell immunophenotypes in perivascular cuffs associated with the inflammatory response to VM antigen was carried out.

Serum samples were obtained from the 22 sheep to evaluate the presence of antibodies against VMV using a commercial test (Elitest®, Hyphen BioMed, Neuville/Oise, France), following the manufacturer's instructions. ELISA results were reported as positive or negative on the basis of the cut-off value calculated following manufacturer's instructions (absorbance 450 nm).

Histological examination revealed that all the 22 studied sheep presented characteristic CNS lesions of VM encephalitis, 9 sheep showed *lymphocytic lesions* and 13 sheep showed *hystiocytic lesions*. Five sheep that showed mild nervous signs (lethargy, tremors, incoordination or mild ataxia of the hindlimbs) presented non-suppurative lymphocytic encephalitis consisting only in perivascular cuffs spread throughout the neuroparenchyma, characterised by the arrangement of round cells in mono- or multiple layers around blood vessels with no or sparse infiltration of the neuropil, named *mild lymphocytic lesions* in this study. Another 4 sheep with more severe nervous clinical signs (progresive ataxia and recumbency) showed these similar mono- and multilayer perivascular cuffs accompanied by a lymphocytic infiltrate in the neuroparenchyma, and these were considered *severe lymphocytic lesions*. Thirteen

animals showed *hystiocytic lesions* with extensive areas of malacia and a non-suppurative hystiocytic infiltrate with evident predominance of large and foamy macrophages. These 13 sheep with hystiocytic encephalitis also showed severe nervous signs of progressive ataxia and recumbency, but five of them with more than 2 week of recumbency. The 22 sheep yielded positive results to the serological tests.

VMV antigen was detected in the brain tissue sections of all the animals in this study, while the control sections were negative. The positive signal was found predominantly in the cytoplasm of the macrophages/microglia located in the CNS lesions. The result of the analysis of the distribution of the viral antigen and the different perivascular cell types associated with the inflammatory response to VMV infection is detailed in Table 1. These results showed clear differences in the phenotypical cellular composition in perivascular spaces in various types of lesion and in the distribution of the VM antigen.

Table 1.

Visna/maedi antigen distribution and cell immunophenotypes forming the perivascular cuff layers.

	Mild lesion		*Lymphocytic lesion*		*Hystiocytic lesion*	
PC[a]	*Monolayer*	*Multilayer*	*Monolayer*	*Multilayer*	*Monolayer*	*Multilayer*
VM antigen	++	++	S	S	+	+
T cell CD3+	+++	+++	+++	+++	+	++
T cell CD8+	+++	++	+++	++	+	++
T cell CD4+	+	++	+	++	+	++
Macrophage	+	+	+	+	+++	++
B cells	S	S	S	S	+	++

[a]*PC: Perivascular cuffs formed by cellular mono- or multiple layers.*

Table 1. Results of semiquantitative analysis of viral antigen and the cell immunophenotypes in perivascular spaces of mild, lymphocytic and hystiocytic lesions, scored as (s) sporadic, (+) few, (++) moderate, and (+++) many.

In *mild lymphocytic lesions*, where perivascular cuffs seemed to be the only damage, with scarce inflammatory response in the neuropil, VM antigen immunoreactivity was detected in these perivascular sleeves. The antigen positive signal was found in the cytoplasm of macrophage-like cells close to the endothelium, and mixed between the cellular sleeves, but it was hardly detected in the neuroparenchyma (Fig. 1). Interestingly, this finding has been observed in this type of lesion alone, so these antigen immunolabelling could correspond to infected perivascular macrophages, or circulating infected monocytes that differentiate into macrophages entering the CNS by this route and allowing viral replication. These observations would confirm the previously proposed models of VMV neuroinvasion, based on the "Trojan horse" pathological mechanism (Georgsson *et al*, 1989; Peluso *et al*, 1985). In this way, in other lentivirus infections such as simian immunodeficiency virus (SIV) and HIV-1, it has been demonstrated that perivascular macrophages are the primary cells productively infected by the virus, resulting in a disruption of the BBB and allowing greater entry of infected cells into the CNS (Kim *et al*, 2003; Persidsky, 1999; Strazza *et al*, 2011; Williams *et al*, 2001b), as could initially occur in VM encephalitis. Thus, most cells (over 90%) which made up these perivascular sleeves were T-cells mixed with scattered macrophages, often close to the endothelium. Specifically, the CD8+ T-cell subpopulation clearly predominated over CD4+ T-lymphocytes in the mono/bilayer perivascular cuffs, but larger multilayer perivascular cuffs showed these cells in similar proportions of 50%. B-cells were only sporadically observed. Thus, it is possible that in mild VM encephalitis, the CD8+ lymphocytes located around blood vessels may be cytotoxic effector cells when they encounter cells presenting viral antigen. Likewise, in SIV encephalitis it has been reported that CD8+ lymphocytes located angiocentrically appear to control the accumulation of infected macrophages in the CNS in an antigenic-specific manner, also with little accumulation of CD4+ lymphocytes (Freel *et al*, 2011; Kim *et al*, 2004). Specifically, this feature has been demonstrated in SIV infection when the viral load was increased, and progression of the disease was accelerated, in animals whose CD8+ T cells were depleted (Schmitz *et al*, 1999; Williams *et al*, 2001a). However, it has previously been suggested that the cellular immune response of CD8+ together with CD4+ T lymphocytes in inflammatory lesions of VM, may not only be directed against the virus but also against self-antigen (Blacklaws, 2012; Torsteinsdottir et al, 2007; Torsteinsdottir et al, 1992), so the presence of CD4+ T-cells together with

CD8+ T-cells in multilayer perivascular cuffs detected in this study could be representing a stage of lesion progression to more severe lymphocytic lesions.

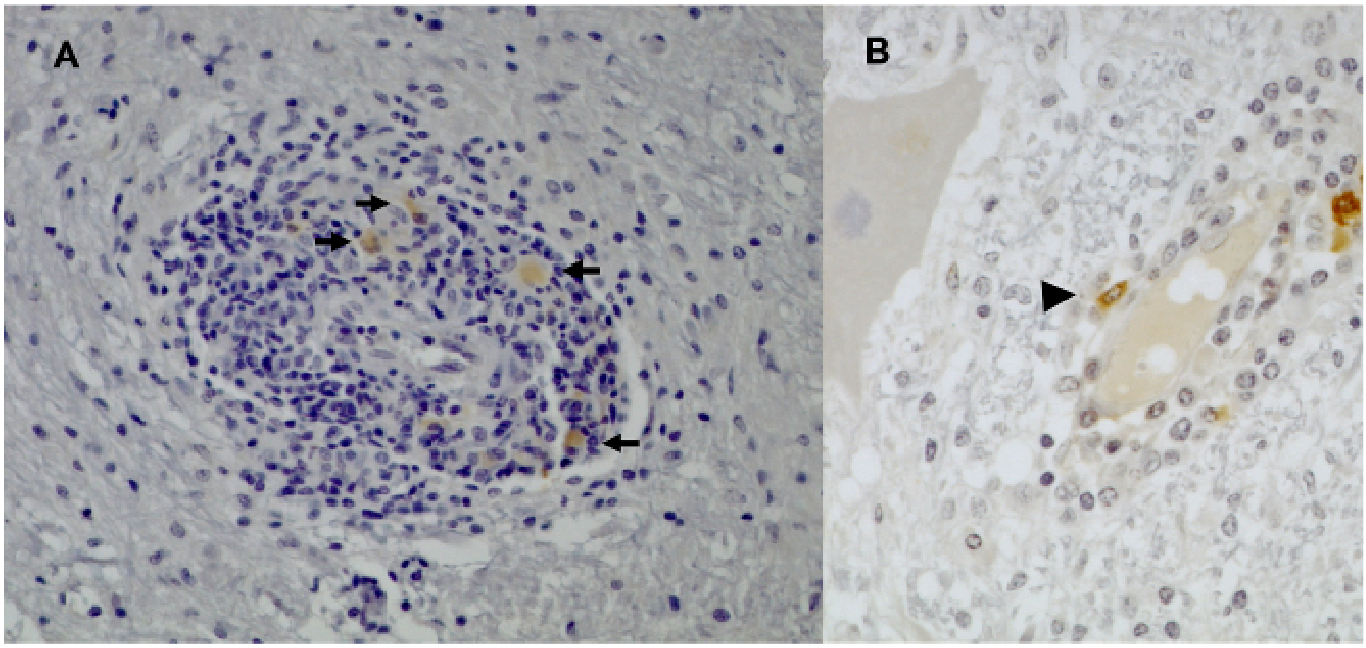

Fig. 1 *Cerebellar peduncles, (A) and (B) mild lymphocytic lesion of VM encephalitis essentially composed of perivascular cuffs with positive antigen signal in macrophages-like cells mixed between the cellular sleeves (arrows). Note that in fig. B the antigen is located close to the endothelium (arrowhead). Anti-p27 VM antigen IHC. (A) x150; (B) x250*

In CNS lesions with a more severe lymphocytic infiltrate of the neuroparenchyma (*severe lymphocytic lesions*) with CD8+ lymphocytes predominance, the presence of antigenic positive signal was very sparse in the perivascular spaces and the neuropil (Fig. 2). Cell immunophenotypes which formed both mono/bilayer and multilayer perivascular cuffs were very similar to the ones previously described in mild lymphocytic lesions, but with a greater abundance of multilayer perivascular sleeves. In severe lymphocytic lesions despite the possible role of the CD8+ T cells in the control of viral replication, this vascular lymphocytic inflammatory response could also cause tissue damage due to dysregulation of the immune response. In this way, in SIV encephalitis lesions, it has also been reported that a large number of activated CD8+ T lymphocytes accumulate abnormally in the brain, resulting in increased concentrations of cytokines to pathological levels (Marcondes *et al*, 2001). Thus, the presence of this lymphocytic infiltrate may be essential in controlling the infection, although it may also

contribute to the progression of the VM lesion. In these perivascular spaces the number of B-cells was very low, so this feature indicates that the humoral immune response would be minimal in this type of lesion, with cellular immunity playing the major role.

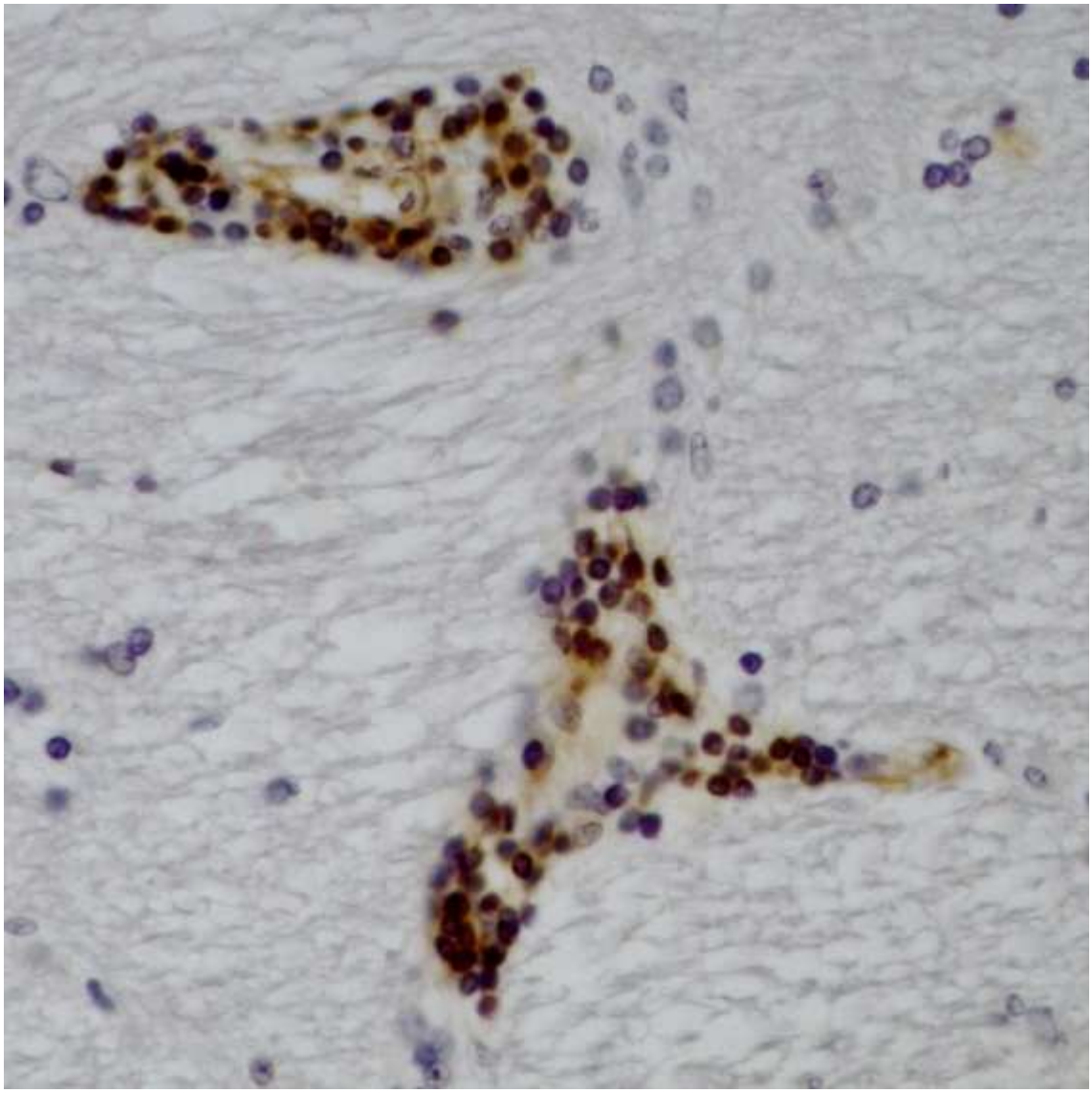

Fig. 2 *Cerebellar peduncles, mononuclear cells around blood vessels in lymphocytic lesions of VM encephalitis, with clear predominance of CD8+-T cell immunoreactivity. Anti-CD8+ T-cell IHC. x250*

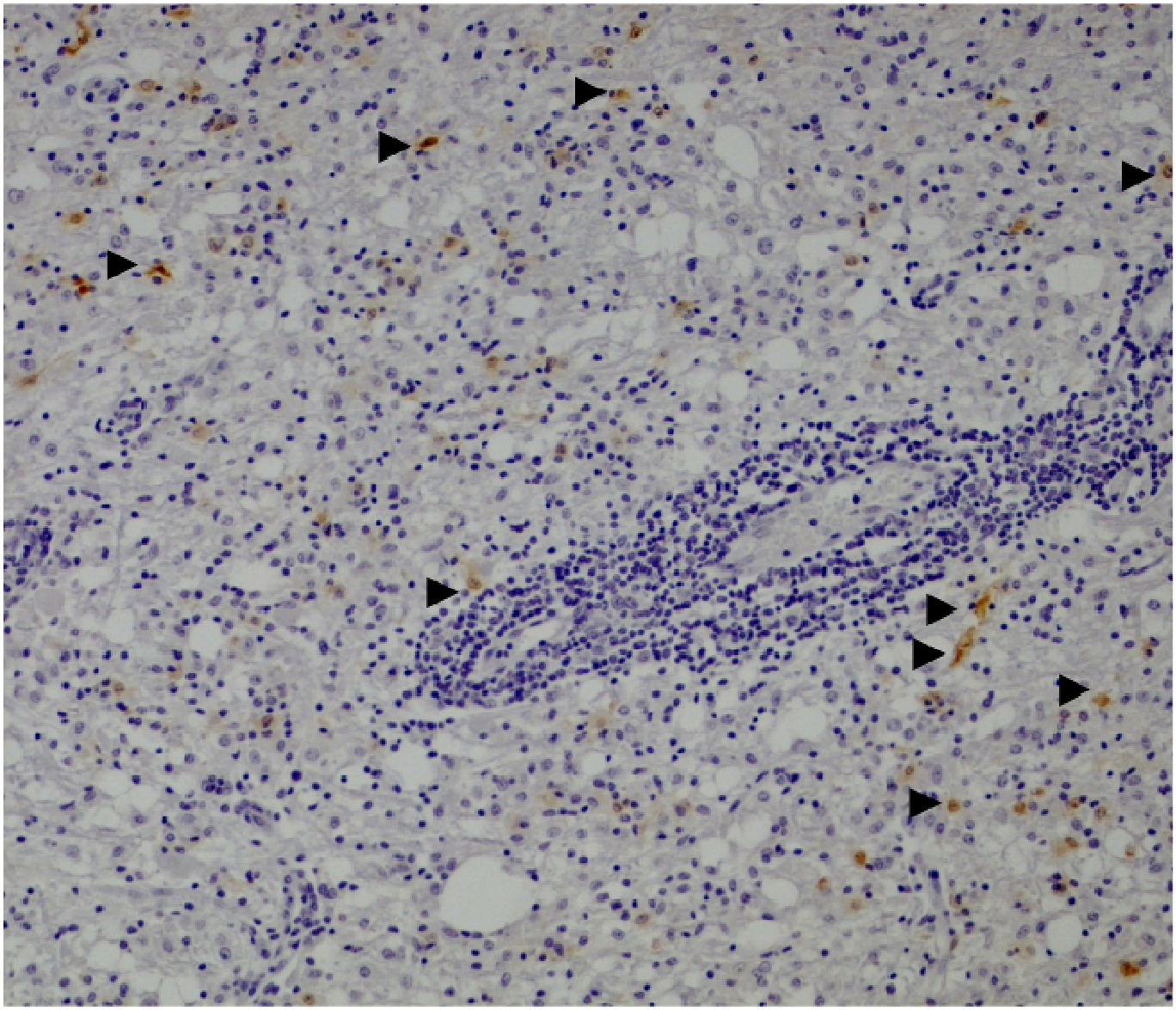

Fig. 3 *Cerebellar peduncles, hystiocytic inflammatory demyelination lesion of VM encephalitis. Antigen positive signal (arrowheads) located in the malacic area. Anti-p27 VM antigen IHC x100*

In particular, examination of lesions in the CNS with hystiocytic infiltration revealed a high number of VMV positive cells in the central areas of the lesion, mixed with malacia (Fig. 3), but this antigen positive signal was rarely detected immediately adjacent to the endothelium or in perivascular cuffs. In these hystiocytic infiltrates, macrophages with a clear cytoplasm ("gitter cells") clearly predominated over other cells such as CD8+ and CD4+ T-cells and B cells. These observations would support the hypothesis of development of VM encephalitis after neuroinvasion of the CNS. Thus, once in the tissue, infected monocytes mature and allow viral replication and recruitment of more infected cells, based on an immune activation in response to viral

antigen that also causes inflammatory infiltration and damage of the CNS tissue (Blacklaws, 2012; Torsteinsdottir *et al*, 2007). This pathological model would explain the abundance of macrophages and viral antigen seen in the parenchyma in the hystiocytic lesion. This way, a slow rate of VM neutralization by antibodies relative to the rate of virus adsorption to cell surface has been suggested as a possible mechanism whereby the virus can spread from cell to cell in the presence of neutralizing antibodies with no free virus release (Thomar, 2005). This feature could explain the persistence and replication of virus in the neuroparenchyma of the hystiocytic lesion in the face of an active immune response. Likewise, the viral induction of apoptosis has been proposed as the major mechanism of cell death occurring during MVV infection (Duval *et al.,* 2002a), and this mechanism is considered to promote cell-to-cell spreading, virus release and stimulation of the immune response (Duval *et al.,* 2002b).

The IHC study of vascular spaces in the areas with abundant malacia of the hystiocytic lesions, often central areas, showed that some blood vessels were completely surrounded by the macrophage infiltration, with few B-cells and T-cells (mainly CD8+ lymphocytes, with CD4+ in lower proportions) adjacent to the endothelium that was occasionally disrupte. In the periphery of the lesion, where multilayer perivascular cuffs were often located, the layers close to the endothelium were mainly formed by T-cells (CD8+ and CD4+, in similar proportions) with around 20% of B-cells, and macrophages behind these layers. This lymphocytic perivascular cuffs may provide a cellular immune response similar to the one described for lymphocytic lesions in response to the spread of the lesion and the consequent endothelial damage. In addition, the observed CD4+ T cells together with B cells tightly packed in these perivascular cuffs may produce an effective utilisation of CD4+ lymphocytic cytokines by B cells, resulting in strong antibody production (Esiri and Gay, 1990), that may be directed against the virus, but also against self-antigens although this is still not clear (Panitch *et al*, 1976; Torsteinsdottir *et al*, 2007). Thus, in these hystiocytic lesions, a non-effective humoral immune response may be involved in the development of tissue damage.

To summerize, this study reinforces and clarifies the prior model of development of VM encephalitis (Blacklaws, 2012; Polledo *et al*, 2011; Torsteinsdottir *et al*, 2007). Thus, the detection of viral antigen in the perivascular spaces, but not in the neuropil, in the described mild lymphocytic lesions may reflect viral neuroinvasion through the

infection of perivascular macrophages or through the entry of infected monocytes into the CNS. Once the infected cells are located in the CNS, viral replication could be controlled by an effective lymphocytic cellular response, but could also progress to severe encephalitis due to dysregulation of the immune response. In the case of lesion progression, immune activation would induce the recruitment and differentiation of more monocytes to macrophages which would enable continuous viral replication and production of cytokines, with an additional non-effective humoral immune response resulting in a severe hystiocytic infiltration and lesion of the CNS. This study showed a spectrum of lesions and immunopathological response closely related to the cells and antigen observed in perivascular spaces, and also a possible relation between the spreading of the virus in the neuroparenchyma and the type and severity of the lesion.

However, the material examined in this study was taken from natural cases of ovine VM with varying degrees of severity which had already shown nervous clinical signs, so further understanding of the inflammatory process in VM encephalitis will require analysis of the inflammatory cells as well as expression of cytokines and endothelial adhesion molecules at well-established stages of the disease, including animals in the subclinical phase.

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TERCER TRABAJO

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SIMPLE CONTROL STRATEGY TO REDUCE THE LEVEL OF MAEDI-VISNA INFECTION IN SHEEP FLOCKS WITH HIGH PREVALENCE VALUES (>90%). *Small Ruminant Research*, Volume 112, Issues 1–3, Pp 224-229

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ABSTRACT

The presence of Maedi-Visna disease in sheep flocks have a significant economic impact, thus the aim of this study was to carry out a serological evaluation of a simple epidemiological control method for ovine Maedi-Visna infection in an intensive dairy flock with high initial seroprevalence. The study population was made up of an initial flock of 197 Spanish Assaf sheep kept in permanent housing using an intensive milking system, and the subsequent five generations of offspring. The control strategy used in this study was mainly based on separating the offspring from the dams after natural intake of colostrum using artificial rearing with milk replacer. Then, replacement groups were formed by these lambs, which were housed in the same shed as the rest of the flock, but in addition, other control measure applied in this study was the isolation of the replacement group from the adults by a barrier (1. 20 metre high metal sheet) that prevented physical contact between them in the housing, although airspace was shared. Then, replacement groups did not joint the adult flock until the onset of first lactation (around 12 months-old). Control measures were first established in 2006 with the second generation offspring, leaving the first replacement group as the control group. Serum samples from all sheep in the flock were screened every six months for antibodies against Maedi-Visna virus using an immunoenzymatic assay. After the application of this control programme, seroprevalence had declined significantly from 93. 1% to 54. 2% in four years. Results from this study indicate that that these control measures may offer a simple alternative for reducing high levels of prevalence in these dairy sheep flocks. Thus, this control strategy is proposed as the first control measures to be taken in flocks with a high prevalence of infection where no other measures could be reasonably applied, followed and expanded by other effective methods when the seroprevalence status of the flock permitting.

Keywords: Maedi-Visna; sheep; control; serology; epidemiology

INTRODUCTION

Maedi-Visna virus (MVV), which belongs to the small-ruminant lentivirus family (SRLV), has a worldwide distribution and is responsible for significant production losses in sheep (Peterhans *et al.,* 2004). Maedi-Visna (MV) disease is characterised by a progressive infection in adult sheep resulting in chronic inflammation of the lungs, mamary gland, joints and central nervous system (Dawson, 1987; Lujan *et al.,* 1991).

A MV prevalence study in the north east of Spain showed that 40. 7% of randomly sampled animals were seropositive, while 97% of the flocks showed seropositive sheeps (Luján *et al.,* 1993). More recently, the prevalence of MV in the north of Spain has been estimated at 77% in intensive flocks, but specially in half the Spanish Assaf intensive flocks this value exceeded 80% (Leginagoikoa *et al.,* 2006b). Several intensive Spanish Assaf flocks present high seroprevalence in Castilla y León (Northwestern of Spain, where this study took place), estimated at 96. 8% in an epidemiological study, particularly in those flocks of dairy sheep which are kept in an intensive indoor farming system (Sotelo, 1998).

Natural lactation (colostrum and milk) and direct contact (oral/respiratory transmission) among animals seem to be the two main sources of infection (Berriatua *et al*., 2003; Blacklaws *et al*., 2004; Álvarez *et al*., 2005; Leginagoikoa *et al*., 2010), and although other routes of Maedi-Visna virus (MVV) transmission, such as vertical and sexual routes, have been reported, they are considered to have limited epidemiological importance (Blacklaws *et al.,* 2004; Broughton-Neiswanger *et al*., 2010; Romero et al., 2010; Cortez-Romero *et al*., 2011). Direct contact exposure to infected sheep, and particularly contact between ewe and lamb, seems to be the most important source of infection and an efficient means of horizontal MVV transmission (Houwers *et al.,* 1989; Berriatua *et al.,* 2003; Álvarez et al., 2005; Leginagoikoa *et al*., 2010), while lactogenic transmission has long been discussed and proposed as a source of MV transmission although making a relatively low contribution to the spread of the disease under natural conditions (Álvarez *et al.,* 2005; Broughton-Neiswanger *et al*., 2010). Consequently, housing practices which determine population density have been strongly associated with the spread of MVV (Leginagoikoa *et al*., 2006a; *Pérez et al*., 2010).

No commercial vaccines are available to control infection, and to date, monitoring programmes have proved to be the only effective tool in controlling infection. During the Icelandic outbreak, the implementation of a compulsory disease monitoring programme associated with slaughter of affected flocks and replacement with MVV-free animals resulted in effective eradication of MVV (Houwers *et al*., 1984; Petursson, 1994). Another control strategy was also used successfully in Iceland, based on dividing each affected flock into two groups, one consisting of the adult sheep and the other comprising of a replacement flock formed by offspring which had been removed immediately after birth (Petursson, 1994). Likewise, other less drastic control programmes have been implemented in many countries (the Netherlands, France, Italy, Germany, for instance.), mainly based on removing serologically positive animals, as well as separating lambs from seropositive dams immediately after birth for rearing on serologically negative or bovine colostrum (Houwers et al., 1984; Houwers et al., 1989).

Sheep culling strategies are feasible in flocks with moderate prevalence (less than 50%), where the number of sheep that become seropositive may be lower than the number of sheep that are annually culled and replaced (Berriatua *et al.*, 2003). However, there are obvious difficulties in applying such strategies, and/or they may be economically unfeasible, in flocks with high seroprevalence as occurs in our region which reach values of over 80%. Hence, our aim was to evaluate a simple and economical control strategy involving minimal changes in flock management that could easily be followed by farmers.

MATERIAL AND METHODS

Study population, management and control strategy of the study flock

The initial study population in 2005 was a dairy flock consisting initially of 197 adult Spanish Assaf ewes from the Farm at the Veterinary School of the University of León (Spain). Sheep were kept in permanent housing, in a tall, well-ventilated housing, with external access to pasture, having a minimum space of 2.5 m2 per animal. Rams from the flock were separated from the ewes except in the breeding season. Males varied depending on the year from 3 to 5, and all of them were adults which came from

other farms and changed throughout the study. The handling of this flock during the study was similar to other dairy intensive flocks from the same region.

This initial adult flock and the following five generations of offspring were studied between 2005 and 2010. Replacement sheep came from the female lambs that were born on the farm. The number of lambs that were kept as replacement animals were 100 (replacement group No. 1), 68 (No. 2), 47 (No.3), 50 (No. 4) and 42 (No. 5), in 2005, 2006, 2007, 2008 and 2009 respectively, not taking into account the immunity status of the dams (seropositive or seronegative to the disease). The rest of the female lambs, which were not included in the replacement groups, and all the male lambs were sold in their first 30 days of age to other farms and were not included in this study. Due to early deaths, the number of sheep that composed each replacement group decreased in the first 18 months of life. The sheep that formed the group of the adult flock each following year were: the adult sheep that have not been sold or have not naturally died from the previous year, together with the replacement group of animals from the previous year (around 12 months old) after their first parturition and beginning of the first lactation.

The handling of the flock was based on a regular management of a dairy intensive flock. The first replacement group from No. 1 was included in this study as a control group and the animals were raised naturally with their dams until 3 months of age. The following groups (No. 2, No. 3, No. 4, No. 5 sheep) were control using the following described control measures: The first one was based on separation of the lambs from the dams after natural intake of colostrum (12-24 hours after birth) and then these lambs were artificially reared with milk replacer. These replacement groups of animals were housed in the same housing as the rest of the flock. But, as a second control measure, these lambs were isolated from the mothers and the adult flock by a barrier (1, 20 metre high metal sheet) with physical contact between them being prevented, although airspace was shared. These replacement animals did not join the adult flock until the onset of the first lactation. In this study, sheep were considered to have reached adulthood at around 12 months of age, after their first parturition. At this time, they joined the adult group, and were kept under the same management, and were also considered part of the adult group in the data of this study.

Gross and histopathological studies

Throughout this study, necropsies were carried out on 78 randomly selected adult sheep submitted to the Pathology Diagnosis Unit at the Veterinary College of León. These animals had naturally died on the farm or they were culled due to evident deterioration of their state of health status and humanly euthanised. Thirty-four sheep came from the initial adult flock, 16 sheep came from No.1 replacement group, 13 from No 2, 7 from No. 3, 4 from No. 4, and 4 from No. 5. Tissue samples from different organs were taken for microscopic examination, with special attention being paid to the lungs, mammary glands, joints and central nervous system. Sample tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin and a histopathological study was carried out. Pathologists were blinded to the serological status of the animals and to the PCR results.

PCR Procedures

Genomic DNA from 9 sheep with characteristic MV lung lesions, and from 4 seronegative sheep with no histologically MV lesions was extracted from lung tissue samples using QIAamp® DNA Mini Kit (QIAGEN). Amplification of LTR regions from these samples was performed as described elsewhere (Benavides et al., 2006a).

Serological testing

Serum samples were obtained every six months from all the animals from six months of age for antibody detection. The presence of antibodies against MVV was evaluated using a commercial enzyme-linked immunosorbent assay (ELISA) (Elitest ®, Hyphen BioMed, Neuville/Oise, France). The test was carried out following the manufacturer's instructions and the results were reported as positive or negative based on the cut-off value calculated as per the manufacturer's instructions (absorbance 450nm). The sensitivity and specificity of this ELISA assay have been estimated as 99. 4% and 99.3%, respectively (Saman et al., 1999; De Andrés et al., 2005). Specifically, the expected Predictive Value Positive and Predictive Value Negative of this ELISA testing in this flock with the initial prevalence of 89% were 99. 9% and 95. 3% respectively.

Statistical analysis

Differences in values of were assesed for significance using the chi-squared analysis. The significance level was set at $p > 0.05$.

RESULTS

The initial number of 197 adult sheep in 2005 which made up the adult flock varied throughout the study to 175 adult sheep in 2006; 214 adult sheep in 2007; 233 adult sheep in 2008; 204 adult sheep in 2009 and finally 190 adult sheep in 2010. The proportion of the flock replaced each year was 53. 1% in 2006, 31. 7% in 2007, 15. 5% in 2008, 23. 0% in 2009, and 22. 1% in 2010.

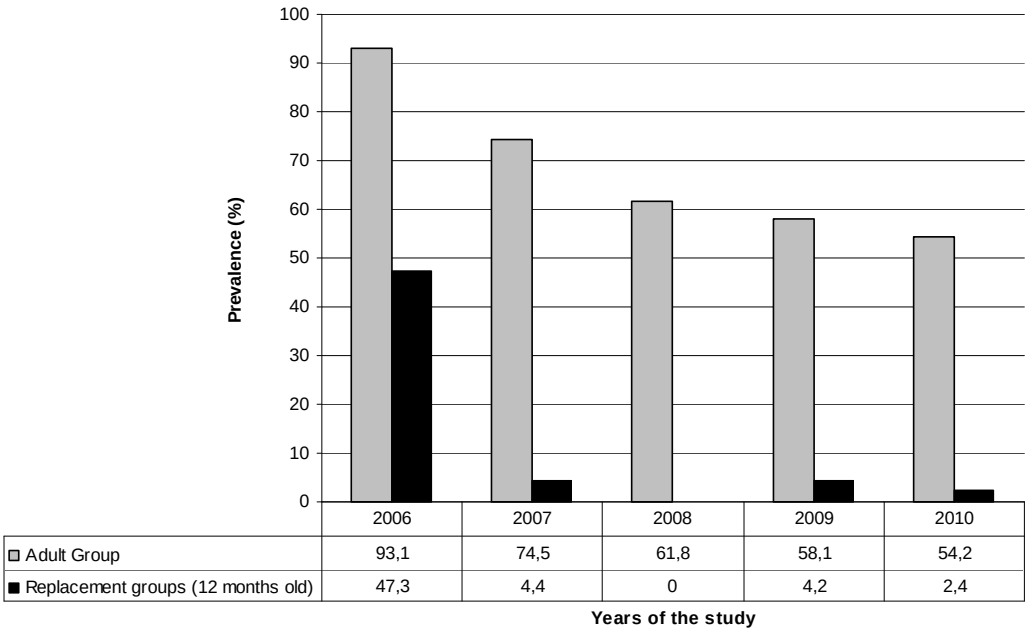
Forty-one of the 78 (52,6%) necropsied animals showed lesions characteristic of MV disease, 38 sheep presenting lung and/or mammary lesions, and 3 sheep with a fatal nervous form and mild lung lesions. Thirty-nine of the 41 animals affected by MV disease yielded positive results in the serological test while two sheep with mild respiratory lesions caused by MV showed negative serological results. Tissue samples of the lung lesions from the 9 animals affected by MV tested by PCR methods yielded positive results, while the PCR results of the 4 seronegative sheep with no MV lesions were negative.

Serological results

The evolution of the prevalence of MV in the adult flock varied from the begining of the study in 2005 which was 89. 0 %. In 2006, MV prevalence in the adult group reached a maximum value of 93. 1%, and decreased over the four years after the onset of the controlled programme to 54. 2% at the end of the study in 2010. These difference in prevalence values of the adult flock from 2006 to 2010 were found to be statistically significant (p= 69, 85). Figure 1 shows the evolution of the prevalence of MV in the adult flock and the respective offspring each year of the study.

The first replacement group, No. 1, used as control with no changes in management and remaining in close contact with the ewes during natural feeding for

their first three months of life, showed an early progressive seroconversion with a prevalence of 47. 3% immediately before reincorporation into the adult flock (at 12 months of age), whereas the subsequent replacement groups that had been under control programme (No. 2, No. 3, No, 4 and No. 5) showed statistically significant differences in prevalence rates of 4.4% (p= 34. 97), 0. 0% (p= 26. 24), 4. 2% (p=28. 52), 2. 4% (p= 26. 28), respectively (Figure 1). Table 1 gives prevalence and number of animals comprising each replacement group and their evolution over the first 18 months of life. In addition, there is an early progressive seroconversion of the No. 1 replacement group from six months of age, whereas the beginning of the marked seroconversion of the No. 2, No. 3, No. 4 replacement groups was posponed until 18 months of age, after they had joined the adult flock. Replacement group No. 5 showed a prevalence of 2. 5% at 12 months of age (last serological test performed in this group of this study).



	2006	2007	2008	2009	2010
Adult Group	93,1	74,5	61,8	58,1	54,2
Replacement groups (12 months old)	47,3	4,4	0	4,2	2,4

Fig. 1 *Evolution of the prevalence of Maedi-Visna infection throughout the years of the study in the adult flock and the consecutive offsprings. Prevalence (y-axis) expressed in percentages. Grey columns indicate the percentage of the prevalence in the adult flock each in the corresponding year throughout the four years of the study. Black columns indicate the prevalence of each replacement group at 12 months old, each in the corresponding year during the four years of the study.*

Specifically, figure 2 shows how the first replacement group reached 40% prevalence in the first 12 months of life, whereas subsequent groups were around 30 months of age before MV prevalence came close to this value.

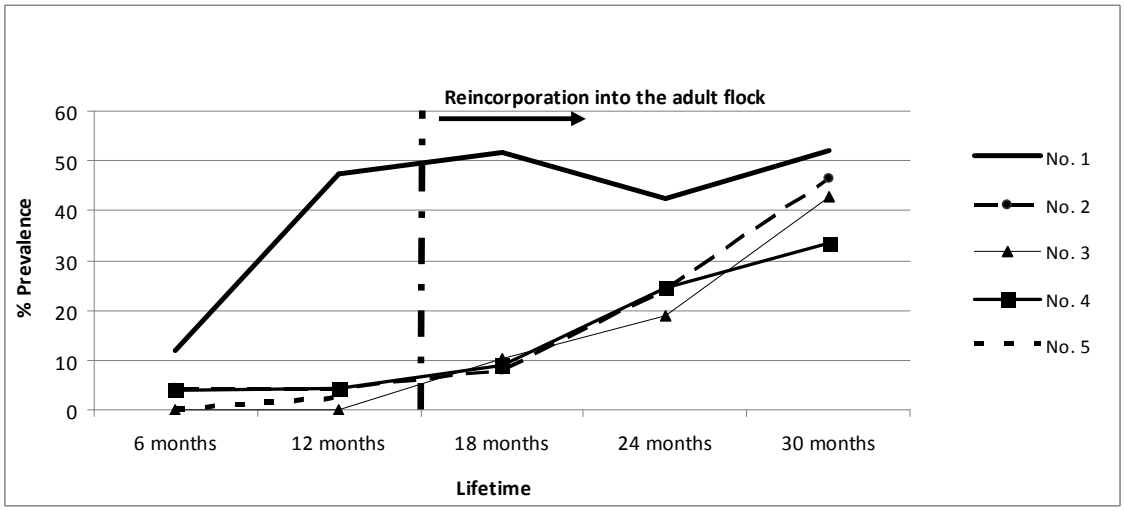


Fig.2 *Evolution of the prevalence in the replacement groups. This figure shows the evolution of the prevalence of infection in percentage (y-axis) throughout the study in the each replacement groups from 6 months to 12 months (No. 5), to 24 months (No. 4) and to 30 months of age (No. 1, No. 2 and No. 3).*

Table 1

Prevalence of each replacement group at 6, 12 and 18 months of age.

	6 months age		12 months age		18 months age	
Replacement group	**Seropositive sheep[a]**	**Prev. (%)**	**Seropositive sheep[a]**	**Prev. (%)**	**Seropositive sheep[a]**	**Prev. (%)**
No.1 (2005)	12/100	12	44/93	47,3	47/91	51,4
No.2 (2006)	3/68	4,4	3/68	4,4	5/64	7,8
No.3 (2007)	0/47	0	0/41	0	4/39	10,2
No.4 (2008)	2/50	4	2/47	4,2	4/44	9,1
No.5 (2009)	0/42	0	1/42	2,4	Not sampled	Not sampled

Table 1. Prevalence evolution of each replacement group thoughout the first 18 months of age. [a] Number of seropositive sheep from the total number of sheep from each replacement group.

Serological results of rams were: 3 seropositive out of 5 in 2005, 4 seropositive out of five in 2006, 4 seropositive out of 5 in 2007, 4 seropositive out of 4 in 2008, 3 seropositive out of 4 in 2009 and 2 seropositive out of 3 in 2010.

DISCUSSION

Due to the economic impact of MV on sheep health and production and the ease with which the virus can spread between and within flocks, control programmes may be the best method for effectively and definitively controlling and even eliminating the virus from an area. Epidemiological knowledge, together with the appropriate diagnostic tools and biosecurity practices should be applied, always taking into account the specific conditions and status of each flock.

Testing and culling are the most commonly used strategies to eradicate infection in other countries, and these have been shown to be successful in flocks with m oderate prevalence of MV disease (<50%) (Houwers *et al.,* 1984; Cutlip and Lehmkuhl, 1986; Houwers *et al*., 1987; Synge and Ritchie, 2010). However, it not economically feasible to apply these control measures to the intensive dairy flocks with high seroprevalence rates (over 80%) detected in our region. Thus, specific control strategies should be established in these flocks in order to reduce these high levels of prevalence to moderate values of around 40-50%, at which point other, stricter control programmes could feasibly be applied.

Recent studies have shown that seroprevalence increased with flock size and the number of days that sheep are housed (Leginagoikoa *et al*., 2006a; Pérez *et al*., 2010; Lago *et al*., 2012), although VMV may not be efficiently airborne over short distances (Leginagoikoa *et al*., 2010). For these reasons, a physical separation between adult sheep and offspring sharing the same housing airspace by means of a simple metal sheet is proposed as an affordable and inexpensive control measure, which does not require any other physical space or housing to keep the replacement animals.

Previous studies have shown that ovine colostrum from seropositive ewes can be a major source of MVV infection but that its overall contribution to seroprevalence under natural conditions is relatively low (Álvarez *et al*., 2005). Moreover, seroconversion has been demostrated to be strongly associated with being born to a seropositive dam, but the risk of seroconversion was similar for lambs fed colostrum and milk from a seropositive or a seronegative dam, independently of the mode of

rearing preweaning (Leginagoikoa *et al.*, 2006b). Likewise, seroconversion has been positively associated with increased contact with infected sheep and with the lifetime MV-serological status of the dam. Consequently, when conditions allow efficient horizontal transmission, the evidence that lactogenic infection increases the risk of MV infection seems to be less important (Berriatua *et al*., 2003). Taking these previous studies into account and in an attempt to modify flock management as little as possible, another affordable measure proposed in this paper is to avoid initial direct contact exposure (oral/respiratory transmission) between the ewe and the lambs (Houwers et al., 1989; Berriatua et al., 2003; Álvarez et al., 2005; Leginagoikoa et al., 2006b) through early separation of the lambs from the ewes within the first 24h of life, after natural consumption of colostrum (for easy handling).

Likewise, vertical and sexual transmission, or indirect contact (insemination, embryo transfer, and other iatrogenic causes) seem to play a less important role in the transmission of the infection (Blacklaws *et al*., 2004), and for this reason no measures were taken in this study to avoid these routes of infection, in order to facilitate flock management. However, these routes of transmission should be considered in order to eradicate the disease.

In this study, the results of PCR and pathological examination indicated the presence of MV infection in the studied flock and its capacity to cause MV disease. The seroprevalence in the entire adult flock decreased from high values of 89. 0 % in 2005 and 93. 1% in 2006, when no possibility existed of implementing standard control programmes, to moderate values of 54. 2% in 2010 after applying the control programme proposed in this study. Since there was no marked change in the number of adult sheep, which ranged from 197 (2005) to 190 (2010), this finding, together with the marked decrease in prevalence rates in the replacement groups over the five years, demonstrates that the decrease of the prevalence rate in the flock was not due to the animal replacement rate.

There were also clear differences between the prevalence rates in the No. 1 replacement group (47. 3%) at one year of age before being reincorporated into the adult flock, and those in the subsequent replacement groups (No. 2, No. 3, No. 4 and No. 5), which did not reach 5%. These data would mean that these first three months of

the lambs´ lifetime are critical in the transmission of the disease, probably due to infection through ingestion of infected colostrum/milk and to the horizontal transmission by the close contact with the mothers. Thus, the avoiding of these ways of tranmission as is put forward in this study results in a minimal early infection of the offspring. These low levels of seroconversion in groups No. 2, No. 3, No.4 and No. 5 could be associated with the ingestion of infected colostrum or vertical transmission (Álvarez *et al*., 2005; Álvarez *et al*., 2006). Thus, the used in breeding of seropositive rams could have contributed to early infections of newborn lambs in this study. Vertical transmission was not considered in the control measures applied in this study in order to simplify handling, but should be taken into account later on in order to eradicate disease from the flock.

In this study it could be detected that the main seroconversion in the control replacement groups No. 2, No. 3, No. 4 occurred after they joined the adult flock and continue along the study, so the infection between adult sheep occurred in an important way. Moreover, the data regarding the delay, from 12 months to 30 months of age, in seroconversion to 40% prevalence in the controlled groups compared with the non-controlled group is very significant, especially in intensive dairy sheep flocks. Since the longevity of these highly productive sheep is on average 4 years of age, and bearing in mind the chronic development of MV disease after infection, manifestation of the disease with the consequent decrease in production as well as premature death would be minimised.

Subsequently, following the decline in prevalence values (54. 2%), other stricter control measures could be established, in order to obtain lower prevalence values. Thus, from this point in which the flock has reached a moderate level of prevalence, it should be possible to reduce flock seroprevalence progressively by selectively culling seropositive sheep and replacing them with offspring from seronegative ewes, as has been previously proposed (Houwers et al., 1984; Cutlip and Lehmkuhl, 1986; Houwers et al., 1987; Berriatua et al., 2003; Synge and Ritchie, 2010). Likewise, isolating these seronegative newborn lambs from the rest of the flock would be very useful (Cutlip and Lehmkuhl, 1986; Reina *et al.,* 2009), and avoiding the ingestion of colostrum by replacing it with bovine colostrum or colostrum from seronegative ewes (Peterhans, 2004). The serological status of the rams should also be taken into account in further

control strategies in order to only use seronegative ones in breeding. Then, a measure which could be incorporated into the final control programme in order to enable early detection of the infected animals and rapid erradication of the infection from the flock could be a combination of serology and polymerase chain reaction (PCR) (Brinkhof *et al.*, 2010). Future objectives include the possibility of officially certifying the MV-free status of flocks as part of a strategy to obtain healthy flocks with respect to MV, enabling farmers to avoid introduction or reintroduction of the infection and the consequent economic loss entailed.

Since successful results were obtained, these simple control measures are proposed as an initial simple control programme on farms with a high level of MV infection where no other measures could reasonably be applied.

CONCLUSION

The aim of this study is to design a MV control programme for implementation in flocks with high prevalence values where the application of other previously described control measures is completely impractical. The results demonstrate that the control programme designed can be applied successfully to reduce high MV prevalence (about 90%) in a dairy sheep flock to more moderate values, and has the potential for extension to larger scale operations. Following this decline in prevalence values, other stricter control measures should be established in order to obtain low prevalence values or even eradicate the infection from the flock.

Conflict of interest statement

None of the authours of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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DISCUSIÓN GLOBAL

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Los dos primeros trabajos incluidos en este estudio describen de forma detallada lesiones nerviosas causadas por el VVM en casos naturales, para contribuir a un mejor conocimiento de la patogenia y patología nerviosa de la enfermedad. Estos trabajos suponen un avance en el conocimiento de la respuesta patológica de cada animal a nivel de tejido nervioso, así como de la posible evolución de la encefalitis en el VM.

En ovino, las formas de presentación más frecuentes de la enfermedad son la respiratoria y mamaria (Lujan *et al.*, 1991), mientras que la forma nerviosa y articular se manifiestan de manera más ocasional (Benavides *et al.*, 2006c; Lujan *et al.*, 1991; Sigurdsson *et al.*, 1957). Sin embargo, en Castilla y León en los últimos años se han diagnosticado frecuentemente encefalomielitis asociada a VM, y se ha calculado que en un 11, 2 % de los animales estudiados con signos clínicos nerviosos, la encefalitis estaba causada por el VVM (Benavides *et al.*, 2006c; Gómez *et al.*, 1999). Estas lesiones nerviosas comparten características patológicas muy similares a las causadas por otros lentivirus como el VIS o el VIH (Leroux *et al.*, 2010; Petursson *et al.*, 1991; Thormar, 2005), por lo que además se trataría de un modelo animal de encefalitis vírica que se podría aplicar para el estudio de estas enfermedades.

La encefalitis causada por VVM ha sido estudiada previamente por otros autores que ya definieron y describieron las manifestaciones clínicas y lesiones asociadas a esta enfermedad (Benavides *et al.*, 2006a; Benavides *et al.*, 2009; Sigurdsson *et al.*, 1957; Sigurdsson and Palsson, 1958). Estos estudios previos han servido de referencia para los trabajos planteados en esta tesis. En concreto, se realizó un estudio inmunohistoquímico de las lesiones tanto para la detección del antígeno del VVM como para la determinación de las poblaciones celulares observadas, y su distribución. Como en las encefalitis víricas la respuesta inmune parece ser responsable de la evolución de la infección y el desarrollo de lesiones (Dorries, 2001), y en la infección por VVM en concreto la inmunidad celular parece jugar un papel muy importante en el desarrollo de lesiones nerviosas y por tanto en la patogénesis del VM, (Torsteinsdottir *et al.*, 1992), nos propusimos llevar a cabo este estudio para caracterizar la poblaciones celulares implicadas en dicha respuesta inmune. En este trabajo, se pudo asignar un patrón lesional a cada animal atendiendo a las lesiones y a las poblaciones celulares presentes

en los mismos. Asimismo, se detectó una estrecha relación entre los infiltrados inflamatorios perivasculares y la presencia y distribución del antígeno vírico.

Las lesiones observadas eran de *tipo linfocítico,* caracterizadas por un infiltrado linfocitario, y de *tipo histiocítico,* con abundacia de células tipo macrófago y células "gitter". Las lesiones se clasificaron en dos subtipos A o B en función de la localización principal y de la extensión.

Se designaron con el *subtipo A* aquellas lesiones que se localizaban principalmente en plexos coroideos o meninges acompañadas o no de leves lesiones en el neuroparénquima, y con el *subtipo B*, en el caso de que el neuroparénquima fuese la región más afectada. Estas diferencias en la localización principal de la lesión nerviosa, se podrían explicar por la vía de entrada del virus al SNC. Así, si el virus penetrara a través del endotelio de plexos coroideos, cabe pensar que desarrollaría las lesiones de tipo coroiditis/meningitis no purulentas, de *tipo A*, si el virus accediera desde vasos al neuroparénquima a través de la barrera hematoencefálica (BHE), causaría una encefalitis no purulenta o lesiones de *tipo B.* Tanto la vía de entrada a través de plexos coroideos, como a través de la BHE, han sido previamente propuestas como rutas de invasión del VVM al SNC (Benavides *et al.*, 2009; Broadwell, 1993; Drevets and Leenen, 2000; Georgsson, 1994; Georgsson *et al.*, 1989; Haase *et al.*, 1977; Peluso *et al.*, 1985).

Otro de los aspectos observados es que en lesiones leves del neuroparénquima de *tipo linfocítico,* el antígeno del VVM se detectó en el citoplasma de células de tipo macrófago alrededor de vasos, entremezclados con numerosos linfocitos-T. Es importante resaltar que solamente en este tipo de lesión, el antígeno vírico se localiza en los espacios perivasculares cercano al endotelio y muy raramente en el neuropilo, lo que sugiere la entrada del virus desde los vasos sanguíneos al SNC a través de la BHE. Además, en este tipo de lesión se detectaron linfocitos-T CD4+ generalmente alrededor de vasos, mientras que los linfocitos-T CD8+ fueron localizados tanto alrededor de vasos como distribuidos por el neuroparénquima tal y como había sido descrito previamente en lesiones nerviosas iniciales por VVM (Torsteinsdottir *et al.*, 1992). Por ello, esta lesión la hemos relacionado con formas lesionales iniciales o latentes de la enfermedad. La presencia de infiltrado linfocitario, tanto en el neuropilo como alrededor

de vasos, podría sugerir una respuesta a la infección por el VVM como ocurre en encefalitis en fase inicial causadas por otros lentivirus como el virus de la AEC, HIV-1 o el virus de la inmunodeficiencia felina (Torsteinsdottir *et al.*, 1992). En estos casos se han descrito infiltrados inflamatorios perivasculares de linfocitos (70%) y células de tipo macrófago (10%), observaciones muy similares a las que se describen en este estudio. Todo ello corrobora la hipótesis de que las lesiones de tipo linfocítico y de limitada extensión serían lesiones iniciales o de latencia. Asimismo, y de acuerdo con lo expuesto previamente, estas lesiones linfocíticas descritas a nivel encefálico podrían corresponderse con fases lesionales iniciales bien conocidas y descritas en otros órganos como glándula mamaria (en VM) o articulaciones (en AEC), donde también se han caracterizado las poblaciones celulares (linfocitos- T CD4+ y CD8+) en el tejido, y especialmente alrededor de vasos (Cordier *et al.*, 1992; von Bodungen *et al.*, 1998).

En concreto, la descripción de los manguitos perivasculares que se observan en este tipo de lesión leve linfocítica coincide con el modelo previamente propuesto del desarrollo inicial de la encefalitis causada por VVM, que indica que la neuroinvasión vírica comenzaría con la activación de las células T a nivel periférico, las cuales serían las primeras en migrar al SNC alterando la BHE (Chebloune *et al.*, 1998). La secreción de linfoquinas facilitaría el paso de otras células infectadas especialmente monocitos/macrófagos al neuropilo. (Georgsson *et al.*, 1989; Peluso *et al.*, 1985).

En infecciones por VIS o VIH-1 se ha propuesto otro modelo de neuropatogénesis basado en que los macrófagos perivasculares serían las primeras células infectadas en llegar al SNC, y alterar la BHE permitiendo la entrada de otras células inflamatorias (Kim *et al.*, 2003; Persidsky, 1999; Strazza *et al.*, 2011; Williams *et al.*, 2001). Según nuestros resultados, el antígeno vírico localizado en macrófagos en los espacios perivasculares podría sugerir que estos provocan un aumento en la permeabilidad de la BHE y favorecen el paso de otras células inflamatorias a los espacios perivasculares del SNC.

A diferencia de estas lesiones iniciales, en lesiones de tipo linfocítico más severas de tipo B donde el parénquima presenta un infiltrado linfocitario más intenso y extenso, apenas se ha localizado señal antigénica del VVM. En este caso, la respuesta inflamatoria se caracterizó claramente por un predominio de linfocitos T CD8+

especialmente en el neuropilo. Ambas observaciones, podrían indicar que la ausencia del antígeno sea debido a la presencia de estos linfocitos-T CD8+ y a su función en la respuesta inmunitaria. A pesar de que su significado no está muy bien definido en el SNC, parece que, como en otros tejidos, puede tener una función citotóxica capaz de inhibir la replicación vírica (Dorries, 2001; Torsteinsdottir *et al.*, 2007). Por ejemplo, en la encefalitis de los simios causada por VIS, se ha descrito que los linfocitos-T CD8+ localizados de forma angiocéntrica junto con la acción antígeno específica de linfocitos T CD4+ en menor número (similar a lo observado en nuestro estudio) parecen controlar la acumulación de células infectadas (Freel *et al.*, 2011; Kim *et al.*, 2004). En este sentido, se ha demostrado que tras una infección vírica del SNC, y en concreto en algunas infecciones por otros lentivirus como VIS, se origina una respuesta inmunitaria de tipo Th1, mediada por linfocitos T CD8, atraídos al punto de inflamación, y capaces de secretar sustancias quimiotácticas que atraerán a otras células inflamatorias (Dorries, 2001; Marcondes *et al.*, 2001). Además, la escasez de células B presentes en estas lesiones linfocíticas, son indicativas de que la respuesta humoral a nivel tisular parece mínima en este tipo concreto de respuesta inflamatoria. Sin embargo, la acción de éstos linfocitos T citotóxicos podría estar no solo dirigida frente al virus, sino también frente a antígenos del propio individuo acentuando aún más la lesión nerviosa (Blacklaws, 2012; Torsteinsdottir *et al.*, 2007; Torsteinsdottir *et al.*, 1992), de forma que a pesar de que estos linfocitos son capaces de ejercer cierto control en cuanto a la replicación vírica, también podrían ser responsables del importante daño tisular que se observa en las lesiones en fases más avanzadas, debido al propio carácter citotóxico de los linfocitos-T CD8+ o la inducción de una respuesta inflamatoria exacerbada (Torsteinsdottir *et al.*, 2007). Por todo ello en la infección por el VVM, la respuesta inmune celular, con predominio de linfocitos-T CD8+ podría ser no sólo importante en el control de la replicación vírica, sino también en el desarrollo y gravedad de las lesiones.

En el caso de las encefalitis de tipo *histiocítico*, tanto en el *tipo A* como *B,* se observaron lesiones graves acompañadas de malacia en el neuroparénquima indicativas de una fase avanzada de la lesión. Este infiltrado histiocitario podría deberse a un incremento excesivo de células inflamatorias y macrófagos, ineficaces en el control de la infección, que propiciaría la replicación vírica (Torsteinsdottir *et al.*, 2007). En lesiones tardías de otras infecciones víricas por lentivirus como VIS o VIH-1 se han

descrito características muy similares a las que se observan en la lesión de tipo histiocítico de este estudio, donde el 90% del infiltrado celular son macrófagos (Kim *et al.*, 2004; Lackner *et al.*, 1991). En nuestro estudio el antígeno vírico se detectó ampliamente distribuido en el neuroparénquima, especialmente en áreas de malacia, pero apenas se comprobó señal antigénica cercana a endotelios vasculares. Estas observaciones corroboran hipótesis anteriores sobre el posible desarrollo de la lesión encefálica en el VM (Blacklaws, 2012; Torsteinsdottir *et al.*, 2007). Así, tras el proceso de neuroinvasión y una vez que las células infectadas llegan al SNC, éstas propiciarán la replicación vírica, provocando en algunos animales el desarrollo de una respuesta inmune ineficaz que atraerá al lugar de la lesión a numerosas células inflamatorias, que liberarán citoquinas y provocarán un importante daño tisular final (Blacklaws, 2012; Torsteinsdottir *et al.*, 2007). En animales con clínica grave de VM, se ha relacionado esta respuesta inmune ineficaz en la eliminación vírica con una presentación defectuosa de antígeno a las células inflamatorias efectoras debido a una deficiencia en las moléculas coestimuladoras B7 (Reina *et al.*, 2007).

Por otro lado, en algunos vasos generalmente localizados en zonas periféricas de estas lesiones histiocíticas, se pudieron observar manguitos perivasculares formados por linfocitos T CD4+ y CD8+ en proporción similar, que podrían interpretarse como una respuesta inmune frente a la progresión de la lesión. No obstante, en estos espacios perivasculares, a diferencia de lo descrito en las lesiones de tipo linfocítico, se observó un número moderado de linfocitos B, cuya interacción efectiva con linfocitos T CD4+ podría resultar en la producción de anticuerpos dirigidos no solo frente al virus, sino también frente a antígenos propios del animal (Esiri and Gay, 1990). En relación con este hecho, en lesiones crónicas artríticas de AEC, también existe un incremento marcado de células B, a diferencia de las lesiones iniciales, asociado a una desviación de la respuesta inmunitaria hacia tipo Th2, de escasa eficacia en la eliminación vírica (von Bodungen *et al.*, 1998; Wilkerson *et al.*, 1995). Por lo tanto, parece probable que la respuesta humoral puede estar implicada en la patogenia, pero sea ineficaz en el control de la infección.

Estas formas linfocíticas e histiocíticas, podrían ser similares a las que se producen en otra enfermedad "lenta", como la paratuberculosis ovina (Clarcke *et al.*, 1996; Pérez *et al.*, 1995), en este caso producido por una micobacteria intracelular

(*Mycobacterium avium sub. paratuberculosis*). En la misma, se han descrito formas *multibacilares*, de claro patrón histiocítico y elevada presencia de bacilos, y formas *linfocíticas* o *paucibacilares*. Relacionada la primera con una respuesta de tipo humoral, y la segunda de tipo celular (Pérez *et al*., 1997; 1999), tal y como podría suceder en los tipos descritos en este trabajo en el VM ovino.

En resumen, los resultados obtenidos en estos dos trabajos confirmaría los modelos e hipótesis previamente propuestos de neuroinvasión y neuropatogénesis vírica del VM (Blacklaws, 2012; Torsteinsdottir *et al*., 2007). En base a estos datos, la neuroinvasión comenzaría con la entrada de monocitos infectados al SNC o por la infección de macrófagos perivasculares. La detección del antígeno vírico en los espacios perivasculares de las lesiones leves linfocíticas apoyaría dicha hipótesis. Una vez que el virus alcanza el neuroparénquima, esté podría ser controlado por una respuesta linfocítica efectiva, representada en este caso por los tipos de lesión *linfocíticos*, o bien progresar a una encefalitis más grave debido a un defecto en la regulación y eficacia de la respuesta inmune, reflejada en los tipos histiocíticos. En este caso los monocitos atraídos al neuroparénquima y diferenciados a macrófagos, facilitarían la replicación vírica y la cronificación de la respuesta inmune, con el consiguiente daño tisular. A todo ello hay que sumar que en esta fase avanzada de lesión, donde se observa un número moderado de células B, se podría además estar desarrollando un respuesta humoral inefectiva de tipo Th2, que agravaría aún más la lesión, y por tanto la enfermedad. Con todo ello, se estaría creando un círculo vicioso donde el mecanismo de patogénesis lesional se basaría en una activación crónica de la respuesta inmunitaria que induciría la diferenciación y atracción de macrófagos y células B a nivel tisular, estimulando la replicación vírica e incrementando la secreción de citoquinas, de forma que atraerían un mayor número de células inflamatorias cerrándose así el círculo (Ebrahimi *et al.*, 2000; Torsteinsdottir *et al.*, 2007).

En conclusión, nuestro trabajo relaciona los tipos lesionales con diferentes etapas de la enfermedad o mecanismos de resistencia frente a la evolución de la misma, de forma que las lesiones de tipo *linfocítico* podrían ser indicativos de fases iniciales o fases latentes de la enfermedad, o de resistencia a la replicación vírica, mientras que las lesiones de tipo histiocítico se relacionarían con lesiones graves y elevada presencia vírica debido a una respuesta ineficaz frente al virus.

El tercer trabajo incluido en este estudio como ya se ha comentado en el planteamiento de este trabajo de tesis, surgió de la necesidad urgente de crear un plan de control de bajo coste económico, de fácil aplicación y eficaz, adaptado a las particularidades de los sistemas de producción y situación de la enfermedad en la Comunidad Autónoma de Castilla y León. En esta región, el sistema de explotación más común entre los ganaderos, es el de explotación lechera intensiva, especialmente entre los ganaderos de raza Assaf española donde existen rebaños con altos niveles de seroprevalencia de rebaño, superior al 80% en la mayoría de las explotaciones. En estos sistemas de manejo el rebaño permanece en estabulación permanente durante todo el año, con los animales en estrecho contacto entre sí.

Las estrategias de control más comúnmente utilizadas con éxito en otros países con niveles de prevalencia mucho más moderados son principalmente: la realización de estudios serológicos de los rebaños, llevado a cabo junto con el sacrificio de animales seropositivos, y la separación de animales formando dos grupos, un rebaño de seropositivos y otro de seronegativos (Cutlip and Lehmkuhl, 1986; Houwers et al., 1987; Houwers et al., 1984; Synge and Ritchie, 2010). Dados los niveles de prevalencia presentes de nuestra región (frecuentemente, mas del 80% y 90% como hemos mencionado) es evidente que los programas de control citados, resultan excesivamente drásticos y económicamente no viables en estos casos.

Por lo tanto, dado las pérdidas económicas que acarrea esta enfermedad, unido a la ausencia de vacunas comerciales en el mercado, resulta necesario la implantación de un método de control adaptado a las necesidades de los rebaño locales para limitar la diseminación de la infección e incluso llegar a conseguir la erradicación de la enfermedad. El objetivo del programa de control propuesto en este trabajo no persigue eliminar la infección del rebaño, sino reducir los altos niveles de prevalencia (de más de 90%) a niveles más moderados alrededor del 50%, que permitan la aplicación de otras medidas de control más estrictas de una manera viable.

Para minimizar la transmisión horizontal en rebaños estabulados una de las medidas tomadas para el control de la enfermedad propuesta y estudiada en este trabajo se basa en evitar el contacto físico entre el grupo de reposición y el rebaño adulto

compartiendo el mismo espacio aéreo dentro de una misma nave. Para conseguirlo, el grupo de hembras adultas y el grupo de reposición fueron separados por una plancha metálica de 120 cm. de altura de manera que no se permitía el contacto físico directo entre ellos. Está demostrado que el VVM parece no transmitirse entre los animales en las distancias cortas (Leginagoikoa et al., 2010). Además, mediante esta separación se evitó el uso compartido de bebederos y comederos, evitándose así la transmisión a través de fómites (Gudnadottir and Palsson, 1965; Villoria et al., 2013). La aplicación de esta medida es muy asequible y no requiere disponer de otras instalaciones adicionales, siendo mínima además la alteración del manejo.

Otra medida propuesta en este estudio como parte de la estrategia de control, consistió en la separación precoz de los corderos de las madres tras el encalostramiento (unas 24-48h después al nacimiento). El contagio entre madre y cordero se considera que se da por dos vías fundamentalmente: por transmisión lactogénica (encalostramiento y lactación natural) o por transmisión horizontal, por contacto estrecho entre madre-cordero (Houwers et al., 1989), y la contribución de cada una al contagio ha sido objeto de controversia (Berriatua et al., 2003b). Se considera que en condiciones naturales la transmisión vía calostro, aunque tiene un papel importante en la epidemiología, su contribución a al contagio madre-cordero es menor que el contacto estrecho entre los mismos (Álvarez et al., 2005). También se ha demostrado que la seroconversión no presenta variación entre dos grupos de animales separados al nacer de la madre que eran alimentados con calostro y leche que provenía de madres seropositivas o seronegativas (Leginagoikoa et al., 2006b). Sin embargo, si se ha comprobado que la seroconversión esta directamente relacionada con el aumento de tiempo que el cordero pasa en contacto directo con la madre infectada, de forma que cuando es posible la transmisión horizontal madre-cordero, la vía de contagio lactogénica toma un papel menos importante. Por ello, se propone tal y como otros autores han sugerido previamente, reducir al mínimo el contacto directo madre-cordero (Berriatua et al., 2003b; Houwers et al., 1989; Leginagoikoa et al., 2006b; Álvarez et al., 2005). Para conseguirlo sin apenas alterar el manejo importantes, en este trabajo se ha propuesto separar las corderas de las madres tras el consumo inicial de calostro (en las primeras 24h de vida), para pasar posteriormente a lactación artificial y permanecer separadas del rebaño adulto hasta el primer parto. En este momento, las hembras de la

reposición se reincorporarán al resto del rebaño facilitándose de esta forma el manejo en la lactación.

Por otro lado, a pesar de que existen otras formas de transmisión de la enfermedad como la vía vertical (intrauterina o transplacentaria), o la vía sexual (Blacklaws et al., 2004), estas no se han considerado en el diseño de este programa de control debido a que la importancia epidemiológica de las mismas es muy baja en relación con las anteriormente mencionadas. Sin embargo, una vez alcanzados unos niveles medios de prevalencia del rebaño, se debería aplicar un programa de control mucho más estricto que tuviera en cuenta tanto la vía de contagio por encalostramiento, como estas últimas vías enumeradas, para la erradicación de la enfermedad.

Los resultados obtenidos demostraron que con la aplicación de estas sencillas medidas propuestas puede reducir los niveles de infección del rebaño. Así en el rebaño de Assaf estudiado, la prevalencia de VM en el grupo adulto descendió desde niveles iniciales de un 93%, a valores moderados de 54 % al final del estudio, donde ya podrían aplicarse otros métodos de control mucho más estrictos sin causar cambios excesivamente drásticos en el manejo. Se comprobó que el descenso en la prevalencia no era debido a un sacrificio masivo de animales seropositivos o a unas tasas de reposición excesivamente altas, ya que el número de animales en el rebaño adulto apenas varió (de 197 madres en 2005 a 190 madres en 2010) y la tasa de reposición experimentó una marcada disminución a lo largo del estudio debido al descenso en los animales adultos de desvieje.

El grupo inicial control del 2005, grupo sobre el que no se tomaron medidas de control presentó al año de edad una seroconversión del 47,3%, mientras que el resto de grupos de reposición sometidos ya al programa de control de la enfermedad no alcanzan un 5% de seroconversión. Este mínimo porcentaje de seroconversión de los grupos controlados de reposición se atribuye al contagio debido al encalostramiento, o bien a la transmisión vertical que se considera responsable de la infección de hasta un 5-8% de los corderos nacidos de madres seropositivas (Álvarez et al., 2005; Álvarez et al., 2006). En este sentido, es necesario enfatizar el dato de que el primer grupo no sometido a medidas de control, presentó a los 18 meses de edad altos de de prevalencia (51%), mientras que los grupos bajo control se mantuvieron entre 7-10%, por lo que la

mayoría de los corderos nacidos de ésta reposición, al menos los correspondientes a la primera paridera, nacieron de madres seronegativas.

Otro dato importante a tener en cuenta que se desprende de este estudio es que en las reposiciones controladas, se demoró hasta cerca de los tres años de edad la seroconversión del 40% de los animales. Este dato resulta muy importante especialmente en rebaños de explotación lechera intensiva, donde la longevidad media de las ovejas es de 4 años de edad, ya que si se retrasa el inicio de la infección y teniendo en cuenta que el desarrollo de la enfermedad es lento, las pérdidas en producción debidas a la misma serán minimizadas.

Los grupos de reposición en el rebaño adulto experimentan una notable seroconversión tras la entrada en el rebaño adulto, lo cual relacionamos directamente con la alta presión de infección a la que se ven sometidos, siendo en este caso el contagio entre animales por transmisión horizontal. De esta forma se demuestra la mayor importancia relativa de este tipo de transmisión frente a otras vías de contagio, así como la infección de animales ya en edad adulta.

Por lo tanto, en este rebaño una vez alcanzados niveles de prevalencia moderados, se podrían aplicar otros métodos de control más estrictos. Así, la siguiente medida adoptar, sería el sacrificio dirigido de animales seropositivos y el reemplazo de éstos por corderas de reposición de madres seronegativas, medida usada con éxito en otros estudios de control (Berriatua et al., 2003b; Cutlip and Lehmkuhl, 1986; Houwers et al., 1987; Houwers et al., 1984; Synge and Ritchie, 2010). Igualmente, a partir de este momento se debería optar por el encalostramiento artificial, bien con calostro bovino o procedente de madres seronegativas o pasteurizado (Peterhans et al., 2004). Una vez que los niveles de infección sean bajos (con valores inferiores a un 20%), se propone la combinación de la serología con métodos PCR para la detección precoz de hembras seropositivas, y el posterior sacrificio de las mismas, para una rápida erradicación de la infección (Brinkhof et al., 2010b).

En resumen, los resultados obtenidos en este trabajo demuestran que es posible reducir altos niveles de infección de VVM (más de 80% de prevalencia) de un rebaño mediante la aplicación de medidas sencillas y de bajo coste, donde la aplicación de los programas de control habituales resultan inviables, para alcanzar

niveles de prevalencia más moderados (alrededor de un 50%) donde ya es factible la aplicación de otras medidas más estrictas de control que permitan alcanzar la erradicación de la enfermedad.

RESUMEN

RESUMEN

El Visna/maedi ovino es una enfermedad vírica de curso lento que ocasiona graves pérdidas económicas y presenta una alta prevalencia en la ganadería ovina de explotación lechera intensiva, especialmente acusada en Castilla y León. En nuestra región, se ha diagnosticado con frecuencia la forma nerviosa de esta enfermedad mientras que en otras zonas es casi inexistente. Por ello, nuestro equipo investigador lleva años trabajando en esta enfermedad, especialmente en la forma nerviosa de la misma, con el objetivo principal de profundizar en el conocimiento sobre diferentes aspectos neuropatogénicos, mediante el estudio detallado de las lesiones encefálicas y los patrones característicos de cada individuo, así como de la migración vírica y propagación de la lesión en el neuroparénquima. Para la elaboración de esta tesis han sido estudiados casos naturales de encefalitis por el VVM remitidos al Servicio de Diagnóstico de Anatomía Patológica de la Facultad de Veterinaria de León, con el fin de identificar y describir los patrones lesionales de la forma nerviosa, y valorar la relación de los mismos con el tipo de respuesta inmune local individual.

En el primer trabajo presentado se realizó una clasificación histopatológica en tipos y subtipos de patrones lesionales basados, en la extensión de la lesión, la localización de la misma y las poblaciones celulares implicadas, comprobando que se podía asignar un patrón lesional característico de cada individuo. Una vez caracterizado el inmunofenotipo celular de las lesiones, se determinó que la existencia de diferentes patrones lesionales podría estar relacionada con la respuesta inmune individual de cada hospedador. Así, se han sido descrito dos tipos principales de lesión: Tipo *linfocítico*, caracterizado por la presencia de un infiltrado de linfocitos con predominio de linfocitos T; y tipo *histiocítico*, en este caso lesiones más severas con extensas áreas de malacia, con numerosos macrófagos, células B y presencia abundante antígeno vírico. El desarrollo de lesiones de tipo histocítico o linfocítico parece podría estar condicionado por una mayor o menor resistencia a la infección o a la progresión de la enfermedad, de forma que en las lesiones linfocíticas con escasa carga viral, la respuesta inmune podría resultar más efectiva frente a la replicación vírica, que las histiocíticas que representan lesiones más graves y con abundante presencia de antígeno vírico, siendo en este caso la respuesta inmune inefectiva. Estas diferencias podrían estar determinando las fases iniciales, latentes asintomáticas y clínicas de la enfermedad.

Siguiendo esta misma línea, en el segundo trabajo, se ha estudiado el papel que podría estar jugando la BHE en la entrada del virus y en la migración de celular, mediante la caracterización de células inflamatorias en los espacios perivasculares. Con el propósito de estudiar las etapas iniciales de neuroinvasión y posterior desarrollo de lesiones de encefalitis, se han caracterizado los infiltrados inflamatorios celulares perivasculares en relación con la distribución del antígeno viral y el patrón lesional observado. A partir de los resultados de estos dos trabajos, se confirmaría la hipótesis propuesta en trabajos previos por Blacklaws (2012) y Torsteinsdottir *et al*. (2007) sobre la neuropatogénesis de la enfermedad. Teniendo estos dos trabajos y los resultados de esta tesis, la lesión nerviosa parece comenzar con la migración vírica dentro de monocitos a través de la BHE o plexos endoteliales o por la infección de macrófagos perivasculares. Tras la invasión vírica del neuroparénquima, la replicación viral a nivel tisular podría estar controlada por una respuesta linfocitaria efectiva, representada por los casos linfocíticos, o bien degenerar en un defecto en la regulación de la respuesta inmune que permitiría la replicación viral, atrayendo un gran número de monocitos manifestándose como lesión de tipo histiocítico. Además de macrófagos se observaron numerosas células B, que parecen relacionarse con una respuesta humoral ineficaz que contribuiría a un agravamiento de la lesión tisular. Además, debido a esta respuesta inflamatoria, los monocitos infectados migrarían de forma continua al neuroparénquima donde se diferenciarían a macrófagos, facilitándose de esta forma la replicación vírica y la cronificación de la respuesta inmune.

La presencia de VM en un rebaño supone pérdidas económicas importantes, especialmente cuando se trata de explotaciones de alta productividad como las de nuestra región. Por ello, el tercer trabajo viene motivado por el contacto con explotaciones donde esta enfermedad muestra altos niveles de prevalencia (93,1%) y causa graves pérdidas económicas. Los ganaderos en esta situación carecen de vacunas efectivas y programas de control adecuados para combatir la enfermedad. Por estos motivos se establece un programa de control adecuado a las necesidades de estas ganaderías con el fin de reducir la prevalencia. Las medidas de control que se proponen en este estudio persiguen minimizar el contagio entre animales, obteniéndose de forma paulatina un descenso de niveles muy altos de prevalencia a moderados, sin alterar apenas el manejo de los animales y con los mínimos costes económicos. La estrategia de control propuesta se basó fundamentalmente en la separación precoz (primeras 24-

48h de vida) de las corderas de reposición de las madres, tras la toma de calostro natural, para posteriormente recibir lactación artificial. Estos grupos de reposición, se mantuvieron en la misma nave separados únicamente por una valla (plancha de metal) que no permitía el contacto físico con el grupo adulto. Los consecutivos grupos de reposición, se reincorporaron al rebaño adulto tras el primer parto en la primera lactación para facilitar el manejo en el ordeño. Para monitorizar la evolución del programa, se sometió a test serológicos a todo el rebaño cada seis meses obteniéndose resultados favorables, de forma que en 4 años la prevalencia de 93, 1%, pasó a valores moderados de 54, 2%. A partir de estos niveles de prevalencia moderados, ya se podrían aplicar otros programas de control más estrictos que se consideran útiles para la eliminación de la enfermedad.

CONCLUSIONES

CONCLUSIONES

- En la forma nerviosa del VM ovino se puede asignar un patrón lesional de forma individual a cada animal, atendiendo a las características de la inflamación y a las poblaciones celulares presentes. Este patrón estaría relacionado con la respuesta inmune individual del hospedador y la mayor o menor presencia de virus en el tejido.

- Se han descrito dos patrones principales de lesión: De tipo *linfocítico*, caracterizadas por la presencia de un infiltrado con predominio claro de linfocitos, y de tipo *histiocítico,* con abundancia de células tipo histiocito y células B. Éstos patrones han sido a su vez subdivididas en dos subtipos A o B en función de la localización principal y de la extensión: *tipo A*, cuando la localización principal o exclusiva es el plexo coroideo/meninge, y *tipo B* cuando es el neuroparénquima.

- Estas diferencias en la localización principal de la lesión nerviosa sugieren dos formas principales de entrada del virus al SNC: Si el virus penetra a través del endotelio de plexos coroideos, se desarrollarían lesiones de tipo coroiditis/meningitis no purulentas, de *tipo A*. Si el virus penetra a través de vasos del neuroparénquima atravesando la barrera hematoencefálica, causaría una encefalitis no purulenta o lesiones de *tipo B.*

- La presencia de partículas víricas es escasa en las lesiones de tipo linfocítico, y abundante en las de tipo histiocítico. Este hecho, unido al componente celular mencionado para cada tipo de lesión, representaría formas de resistencia o de respuesta ineficaz frente a la replicación vírica respectivamente. Estos tipos lesionales se relacionarían con diferentes etapas de la enfermedad o con mecanismos de resistencia frente a la evolución de la misma.

- Existe una estrecha relación entre las características de los infiltrados inflamatorios perivasculares y la presencia y distribución del antígeno vírico.

Una vez el virus alcanza el neuroparénquima, esté podría ser controlado por una respuesta linfocítica efectiva, o bien progresar a una encefalitis más grave debido a un defecto en la regulación y eficacia de la respuesta inmune.

- La información obtenida en estos dos trabajos confirmaría las sugerencias o hipótesis propuestas previamente de neuroinvasión y neuropatogénesis vírica del VM (Blacklaws, 2012; Torsteinsdottir et al., 2007).

- Evitar el contacto físico entre el grupo de reposición y el rebaño adulto dentro de una misma nave reduce la prevalencia de la infección. Esta separación impide el uso compartido de espacio, bebederos y comederos, evitándose así la transmisión directa y a través de fómites.

- Mediante la aplicación del programa de control propuesto se ha logrado reducir altos niveles de prevalencia de VM (93,1% de prevalencia) a niveles más moderados (54,2%). Estas medidas apenas alteran el manejo del rebaño y no requieren gastos adicionales. En esta situación sería ya factible la aplicación de otras medidas más estrictas que podrían conducir a la erradicación de la enfermedad.

- Los grupos de reposición en el rebaño adulto experimentan una notable seroconversión tras la entrada en el rebaño adulto por transmisión horizontal. Esto demuestra la mayor importancia relativa de esta vía de infección frente a otras vías de contagio, así como la importancia de la infección de ovinos en edad adulta.

SUMMARY

SUMMARY

Ovine Visna/Maedi is a slow developing viral disease which causes severe economic losses and shows a high prevalence in intensive milk-producing sheep flocks, mainly in the Castilla y León region. In this region, the nervous form of this disease has been diagnosed while in other regions it hardly exists. For this reason, our research team have been working on this illness for many year, ,mainly on the nervous form ,with the main aim of obtaining an in-depth knowledge of the different neuropathogenic aspects through a detailed study of the nervous lesions and the characteristic patterns of each individual sheep as well as the viral migration and propagation of the lesion in neuroparenchyma. In order to elaborate this thesis, natural cases of encephalitis caused by VVM submitted to the Pathology Diagnosis Service at the School of Veterinary Medicine in León were studied with the aim of identifying and describing the lesion patterns of the nervous form and evaluating the relationship of these with the type of local individual immune response.

In the first study presented a histopathologic classification in types and subtypes of lesion patterns was carried out based on the extension of the lesion , its location and the cell populations involved showing that a characteristic lesion pattern could be assigned to each individual. Once the immunophenotypes of the lesions were characterized, it was determined that the existence of the different lesion patterns could be related to the individual immune response of each host sheep. Thus, two main lesion types have been described: lymphocytic type, characterized by the presence of an infiltrate with T-cells predominating, and hystiocytic type, in this case lesions were more severe with extensive malacic areas, with various macrophages, B cells and abundant viral antigen presence. The development of hystiocytic or lymphocytic type lesions would seem to be conditioned by a greater or lesser resistance to the infection or to the progression of the disease. This way in the lymphocytic lesions, which have scarce viral charge, the immune response would be more effective as opposed to the viral replication. On the other hand, the immune response would be defective in hystiocytic lesions with abundant viral antigen. These differences could determine the initial, asymptomatic, latent and clinical phases of this disease.

Following these lines, the role of blood-brain barrier could play in the entry of the virus and the cell migration was studied using the characterization of the inflammatory cells in the perivascular cell sleeves in the second study. With the aim of studying the initial neuroinvasion stages and the later development of the encephalitis, the perivascular cuffs were characterized with regard to the distribution of the viral antigen and lesion type observed. From the results of these two studies, the hypothesis put forward in previous studies by Blacklaw (2012) and Torsteinsdottir *et al*. (2007) on the neuropathogenesis of the disease can be confirmed. These two studies together with the results of this thesis show that the nervous lesion would seem to begin with viral migration in mocytes through the blood-brain barrier or choroid plexus, or even by the infection of the perivascular macrophages. After the viral invasion of the neuroparenchyma, the viral replication at tissue level could be controlled by an effective lymphocytic immune response represented by the lymphocytic types or by the degeneration of a defect in the control of the immune response which would allow for the viral replication attracting a large number of monocytes shown as hystiocytic lesion. Various B cells were observed as well as macrophages in hystiocytic lesion, which seem to be related to an ineffective humoral response and would contribute to a worsening of the tissue lesion. Due to this inflammatory response, the infected monocytes also migrate in a continuous way to the neuroparenchyma, where they are different to macrophages, thus facilitating the viral replication and the chronification of the inflammatory immune response.

The presence of VM in the flock entails important economic losses mainly when dealing with high-producing farms like those in our regions. For this reason the third study was motivated by the contact with flocks where this disease shows high levels of prevalence (93.1%) and causes serious economic losses. The farmers in this situation lack efficient vaccines and adequate control programmes to fight this disease, for this reason a control programme suitable for the needs of the se farmers has to be established with the aim of reducing prevalence. The control measures which are proposed in this study try to minimize it from being contagious in animals, obtaining a slow but sure way a decrease from the high levels of prevalence to a moderate one without hardly altering the management of the animals and with minimum economic costs. The control strategy proposed is mainly based on the early separation (the first 24-48h of life) of the lambs from the mothers after taking natural colostrum and later to

receive artificial lactation. These replacement groups were kept in the same building separated only by a metal barrier which did not permit contact with the adult group. The consecutive replacement groups were reincorporated into the adult flock after the first birth in the first lactation to facilitate milking management. In order to monitor the evolution of the programme, the whole flock was submitted to serological tests every six months obtaining favourable results in such a way that the 93.1% prevalence fell to moderate values of 54.2% in four years. From these moderate prevalence levels, other stricter control programme can be applied and which now could be focused on the eradication of this disease.

CONCLUSIONS

CONCLUSIONS

- In the ovine MV nervous form, a lesion pattern can be assigned in an individual way to each animal, paying special attention to the inflammatory characteristics and to the cell populations present. This pattern could be related to the individual immune response of the host and to the greater or lesser presence of virus in the tissue.

- The two main lesion patterns have been described: lymphocytic type, characterized by the presence of an infiltrate with clear predominance of T-cells, and histyocytc type with an abundance of hystiocytic type cells and B cells. These patterns were at the same time subdivided into two subtypes A or B taking into account the main localization and extension of the lesion: Type A when the main or exclusive lesion is in the chorid plexus and type B when it is neuroparenchyma.

- These differences in the main localization of the nervous lesion show two main ways of virus entry into central nervous system: If the virus penetrates through the endothelium of the choroid plexus, lesions type A would develop, and If the virus penetrates through the vessels of the neuroparenchyma migrating through the blood-brain barrier, it would cause an encephalitis or type B lesion.

- The presence of viral particles is scarce in lymphocytic type lesions and abundant in the hystiocytic types. This fact, together with the previously mentioned cell component for each lesion type could represent forms of resistanc or of ineffective response as opposed to the virus replication, respectively. These lesion types could be related to the different stages of the illness or to resistance mechanism as opposed to its evolution.

- There exists a close relationship between the characteristics of the inflammatory infiltrate and the presence and distribution of the viral antigen. Once the virus reaches the neuroparenchyma, it can be controlled by an effective lymphocytic

immune response or even progressing to more severe encephalitis due to a defect in the regulation and the efficiency of the immune response.

- The information obtained in these two studies would confirm the previously proposed suggestions or hypothesis of neuralinvasion and viral neuropathogenesis of VM (Blacklaws t al, 2012; Torsteinsdottir *et al*. 2007).

- Avoiding physical contact between the replacing-groups and the adult flock in the same building reduces prevalence of the infection. This separation stops the shared use of space, drinking troughs and feeding boxes, thus avoiding direct transmission through fomites.

- By using the application of the proposed control programme, a reduction of high prevalence levels of VM (93.1% prevalence) to more moderate levels (54.2%) was achieved. These methods hardly alter the management of the flock and do not require any additional expense. The application of other stricter methods which could lead to the eradication of this disease would be practical in this situation.

- The replacement groups in the adult flock underwent a marked seroconversion after entry into the adult flock by horizontal transmission. This shows a greater relative importance of this via of infection as opposed to other ways of transmission as well as the importance of the infection in adult ovines.

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ANEXO I. Otras publicaciones con participación de esta tesis

ANEXO II. Otras publicaciones con participación de esta tesis

Visna/Maedi virus genetic characterization and serological diagnosis of infection in sheep from a neurological outbreak. Glaria I, Reina R, Ramírez H, de Andrés X, Crespo H, Jauregui P, Salazar E, Luján L, Pérez MM, Benavides J, Pérez V, Polledo L, García-Marín JF, Riezu JI, Borrás F, Amorena B, de Andrés D. *Vet Microbiol. 2012 Mar 23; 155(2-4): 137-46. doi: 10.1016/j.vetmic.2011.08.027.*

An extensive outbreak characterized by the appearance of neurological symptoms in small ruminant lentivirus (SRLV) infected sheep has been identified in Spain, but the genetic characteristics of the strain involved and differential diagnostic tools for this outbreak remain unexplored. In this work, 23 Visna-affected naturally infected animals from the outbreak, 11 arthritic animals (both groups presenting anti-Visna/Maedi virus serum antibodies), and 100 seronegative animals were used. Eight of the Visna-affected animals were further studied post-mortem by immunohistochemistry. All had lesions in spinal cord, being the most affected part of the central nervous system in six of them. A representative strain of the outbreak was isolated. Together with other proviral sequences from the outbreak the virus was assigned to genotype A2/A3. In vitro culture of the isolate revealed that viral production was slow/low in fibroblast-like cells but it was high in blood monocyte-derived macrophages. The long terminal repeat (LTR) of the viral genome of this isolate lacked an U3-duplication, but its promoter activity in fibroblast-like cells was normal compared to other strains. Thus, viral production could not be inferred from the LTR promoter activity in this isolate. Analysis of the viral immunodominant epitopes among SRLV sequences of the outbreak and other known sequences allowed the design of a synthetic SU peptide ELISA that detected the Visna affected animals, representing a tool of epidemiological interest to control viral spread of this highly pathogenic strain.

Study of compartmentalization in the visna clinical form of small ruminant lentivirus infection in sheep. Ramírez H, Reina R, Bertolotti L, Cenoz A, Hernández MM, San Román B, Glaria I, de Andrés X, Crespo H, Jáuregui P, Benavides J, Polledo L, Pérez V, García-Marín JF, Rosati S, Amorena B, de Andrés D. *BMC Vet Res. 2012 Jan 26;8:8. doi: 10.1186/1746-6148-8-8.*

Background: A central nervous system (CNS) disease outbreak caused by small ruminant lentiviruses (SRLV) has triggered interest in Spain due to the rapid onset of clinical signs and relevant production losses. In a previous study on this outbreak, the role of LTR in tropism was unclear and env encoded sequences, likely involved in tropism, were not investigated. This study aimed to analyze heterogeneity of SRLV Env regions--TM amino terminal and SU V4, C4 and V5 segments--in order to assess virus compartmentalization in CNS. Results: Eight Visna (neurologically) affected sheep of the outbreak were used. Of the 350 clones obtained after PCR amplification, 142 corresponded to CNS samples (spinal cord and choroid plexus) and the remaining to mammary gland, blood cells, bronchoalveolar lavage cells and/or lung. The diversity of the env sequences from CNS was 11.1-16.1% between animals and 0.35-11.6% within each animal, except in one animal presenting two sequence types (30% diversity) in the CNS (one grouping with those of the outbreak), indicative of CNS virus sequence heterogeneity. Outbreak sequences were of genotype A, clustering per animal and compartmentalizing in the animal tissues. No CNS specific signature patterns were found. Conclusions: Bayesian approach inferences suggested that proviruses from broncoalveolar lavage cells and peripheral blood mononuclear cells represented the common ancestors (infecting viruses) in the animal and that neuroinvasion in the outbreak involved microevolution after initial infection with an A-type strain. This study demonstrates virus compartmentalization in the CNS and other body tissues in sheep presenting the neurological form of SRLV infection.

Mannose receptor may be involved in small ruminant lentivirus pathogenesis.Crespo H, Jauregui P, Glaria I, Sanjosé L, Polledo L, García-Marín JF, Luján L, de Andrés D, Amorena B, Reina R. *Vet Res. 2012 May 16;43(1):43. doi: 10.1186/1297-9716-43-43.*

Thirty-one sheep naturally infected with small ruminant lentiviruses (SRLV) of known genotype (A or B), and clinically affected with neurological disease, pneumonia or arthritis were used to analyse mannose receptor (MR) expression (transcript levels) and proviral load in virus target tissues (lung, mammary gland, CNS and carpal joints). Control sheep were SRLV-seropositive asymptomatic (n = 3), seronegative (n = 3) or with chronic listeriosis, pseudotuberculosis or parasitic cysts (n = 1 in each case). MR expression and proviral load increased with the severity of lesions in most analyzed organs of the SRLV infected sheep and was detected in the affected tissue involved in the corresponding clinical disease (CNS, lung and carpal joint in neurological disease, pneumonia and arthritis animal groups, respectively). The increased MR expression appeared to be SRLV specific and may have a role in lentiviral pathogenesis.

An insight into a combination of ELISA strategies to diagnose small ruminant lentivirus infections. de Andrés X, Ramírez H, Bertolotti L, San Román B, Glaria I, Crespo H, Jáuregui P, Minguijón E, Juste R, Leginagoikoa I, Pérez M, Luján L, Badiola JJ, Polledo L, García-Marín JF, Riezu JI, Borrás-Cuesta F, de Andrés D, Rosati S, Reina R, Amorena B. *Vet Immunol Immunopathol. 2013 Apr 15;152(3-4):277-88. doi: 10.1016/j.vetimm.2012.12.017.*

A single broadly reactive standard ELISA is commonly applied to control small ruminant lentivirus (SRLV) spread, but type specific ELISA strategies are gaining interest in areas with highly prevalent and heterogeneous SRLV infections. Short (15-residue) synthetic peptides (n=60) were designed in this

study using deduced amino acid sequence profiles of SRLV circulating in sheep from North Central Spain and SRLV described previously. The corresponding ELISAs and two standard ELISAs were employed to analyze sera from sheep flocks either controlled or infected with different SRLV genotypes. Two outbreaks, showing SRLV-induced arthritis (genotype B2) and encephalitis (genotype A), were represented among the infected flocks. The ELISA results revealed that none of the assays detected all the infected animals in the global population analyzed, the assay performance varying according to the genetic type of the strain circulating in the area and the test antigen. Five of the six highly reactive (57-62%) single peptide ELISAs were further assessed, revealing that the ELISA based on peptide 98M (type A ENV-SU5, consensus from the neurological outbreak) detected positives in the majority of the type-A specific sera tested (Se: 86%; Sp: 98%) and not in the arthritic type B outbreak. ENV-TM ELISAs based on peptides 126M1 (Se: 82%; Sp: 95%) and 126M2 0,65 0.77 (Se: 68%; Sp: 88%) detected preferentially caprine arthritis encephalitis (CAEV, type B) and visna/maedi (VMV, type A) virus infections respectively, which may help to perform a preliminary CAEV vs. VMV-like typing of the flock. The use of particular peptide ELISAs and standard tests individually or combined may be useful in the different areas under study, to determine disease progression, diagnose/type infection and prevent its spread

ANEXO II. Trabajos en formato original

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INFECTIOUS DISEASE

Patterns of Lesion and Local Host Cellular Immune Response in Natural Cases of Ovine Maedi-Visna

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Summary

This study investigates the nervous form of ovine maedi-visna by histological and immunohistochemical techniques. The aim was to study the lesion types and the local cellular immune response related to each lesion type, and the possible relationship between these parameters. Thirty-four Assaf ewes were studied, 29 of which had shown nervous signs. Microscopical lesion patterns were described according to location, extent and predominance of inflammatory cell type. Immunohistochemical labelling of T cells ($CD3^+$, $CD4^+$, $CD8^+$ and cells expressing the γδ form of the T-cell receptor), B cells and macrophages revealed clear differences between the lesion patterns. Two main lesion types were described. Lymphocytic lesions had areas of mild-moderate injury characterized by a predominance of infiltrating T cells. Histiocytic lesions were more severe and had extensive areas of malacia and dominant infiltration by macrophages and B cells. Each animal had a unique lesion pattern and these differences could be due to individual resistance to the progression of infection. The lymphocytic lesions appear to represent initial or latent phases of slow progression, in which the animal presents some natural resistance to the infection. The histiocytic pattern may reflect a poor immune response or a greater virulence of the viral strain.

Keywords: immunopathology; lentivirus; maedi-visna; sheep

Introduction

Maedi-visna (MV) is a slow, progressive multisystemic disease of sheep that is caused by the maedi-visna virus (MVV). MVV is a retrovirus of the genus *Lentivirus* that belongs to the small-ruminant lentivirus (SRLV) group and is distributed worldwide. The SRLV group includes all strains of MV and caprine arthritis encephalitis virus (CAEV) (Zanoni, 1998). The disease commonly affects adult sheep and leads to a multisystemic syndrome characterized by progressive weight loss and chronic non-purulent lung, mammary gland, joint and central nervous system (CNS) inflammation (Dawson, 1987; Cutlip *et al.*, 1988).

In Spain, MV is considered a widespread disease, although the data vary between studies (Lujan *et al.*, 1991, 1993; Benavides *et al.*, 2006c; Leginagoikoa *et al.*, 2006; Alba *et al.*, 2008). SRLV seroprevalence in the north of Spain has been estimated at 77% in intensive flocks, but half of flocks of the Spanish Assaf breed have a prevalence of >80% (Leginagoikoa *et al.*, 2006). These flocks are under a particular type of management whereby dairy sheep are kept in intensive indoor farming facilities. Several Spanish Assaf flocks in one region have reached an estimated seroprevalence of 96.8% (Sotelo, 1998).

The disease most commonly presents in respiratory or mammary form, while the nervous form is sporadic (Sigurdsson *et al.*, 1957; Lujan *et al.*, 1991; Benavides *et al.*, 2006a, 2009). Recently, numerous cases of the nervous form (even in animals 4–6 months of age) have been diagnosed, especially in the Assaf breed (Benavides *et al.*, 2007; Leginagoikoa *et al.*, 2010). Nervous signs include hindlimb weakness and

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ataxia, hypermetria or paralysis usually leading to recumbency, although the animal remains alert and responds to external stimuli (Sigurdsson *et al.*, 1957; Christodoulopoulos, 2006; Benavides *et al.*, 2006c, 2009). Lesions in the brain or spinal cord are usually not apparent grossly, but occasionally severe brain injuries are identified as liquefactive areas in the white matter of the encephalon and wedge-shaped areas of malacia in the spinal cord (Sigurdsson *et al.*, 1957; Sigurdsson and Palsson, 1958; Benavides *et al.*, 2006a, 2009).

Microscopically, chronic non-purulent meningoencephalomyelitis, usually accompanied by demyelination, is a common finding. Mononuclear infiltration of the choroid plexus that sometimes results in the development of ectopic lymphoid follicles may also occur (Cutlip *et al.*, 1979). Histological lesions have been classified into three main patterns (Benavides *et al.*, 2009) according to the location of the lesion and regardless of the cellular population comprising the lesion. In the vascular pattern, mononuclear cells are arranged around blood vessels forming perivascular cuffs. In the infiltrative pattern a non-purulent infiltration of the neuroparenchyma accompanies perivascular cuffing. Demyelination is the main feature of the malacic pattern. More than one of these patterns may be identified in any individual animal.

The importance of cell-mediated immunity in relation to the severity of the CNS lesions has been demonstrated in previous studies of sheep infected intracerebrally with MVV (Torsteinsdottir *et al.*, 1992); however, the nature of the cellular infiltration in these CNS lesions has not been determined. In contrast, there are reports characterizing the cellular infiltrates in the respiratory and mammary forms of the disease (Lujan *et al.*, 1991; Cordier *et al.*, 1992; Christodoulopoulos, 2006).

The aim of the present study was to further characterize specific lesion patterns and the nature of infiltrating cellular populations in Assaf sheep with the neurological form of MV.

Materials and Methods

Animals

Thirty-four Assaf sheep submitted to the Pathology Diagnostic Service of the Veterinary School of León were investigated. The animals came from different flocks subjected to the intensive indoor farming system widespread in the northwest of Spain. Twenty-two animals were adults (>2 years of age), nine sheep were 1–2 years of age and three were 4–6 months of age.

Histopathology

Tissue samples were taken for microscopical examination, including from the lung, mammary gland, joints and CNS. CNS samples were obtained systematically, taking sections of the cortex, diencephalon, corpus callosum, hippocampus, midbrain, cerebellar cortex, pons and cerebellar peduncles, medulla oblongata and cervical, thoracic and lumbar spinal cord. Samples were fixed in 10% neutral buffered formalin for 24 h at room temperature. Sections (3 μm) were stained with haematoxylin and eosin (HE).

Immunohistochemistry

CNS tissues from 16 sheep were immersed in zinc fixative salts (ZFS; 0.5% zinc chloride, 0.5% zinc acetate in 0.05% Tris buffer with 0.1 M calcium acetate, pH 7.4; González *et al.*, 2001) for 24 h at 4°C. After fixation, samples were dehydrated through graded alcohol and embedded in paraffin wax. Sections (3 μm) were subjected to immunohistochemistry (IHC) using a polymer-based detection system (EnVision+ System Labelled Polymer-HRP anti-mouse or anti-rabbit; Dako, Glostrup, Denmark). Sections were dewaxed and rehydrated and endogenous peroxidase activity was blocked with H_2O_2 3% in methanol for 30 min. Non-specific binding was blocked with normal goat serum diluted 1 in 20 in Tris buffered saline (TBS) with bovine serum albumin (BSA) for 20 min. Slides were incubated overnight with primary antibody (Table 1). Subsequently, the sections were incubated for 30 min using the EnVision+ system and developed with a solution of 3, 3′diaminobezidine (DAB) (Vector Laboratories, Burlingame, California, USA). The slides were counterstained with haematoxylin and mounted. The specificity of the technique was evaluated by negative controls (omission of primary antibody and substitution of primary antibody by non-immune serum) and positive controls (lymph node sections and positive samples of tissue previously tested as positive for MVV). Tissue samples from an animal without

Table 1
List of primary antibodies

Antibody specificity (clone)	*Host species*	*Source*
CD3 (A0452)	Rabbit	Dako, Glostrup, Denmark
CD4 (17D1)	Mouse	VMRD, Pullman, Washington, USA
CD8 (AB4055)	Rabbit	Abcam, Cambridge, UK
CD79 αcy (HM57)	Mouse	Dako
CD68 (KP1)	Mouse	Dako
γδ TCR (CC15)	Mouse	Serotec, Kidlington, UK

characteristic lesions of MV and with negative serological and polymerase chain reaction (PCR) tests were included as a further negative control.

Results were scored subjectively as: ±, few scattered positive cells; +, moderate number of positive cells; ++, large number of positive cells; +++, predominant cellular population.

Formalin-fixed tissue samples from 24 animals were also tested by immunohistochemical techniques to determine expression of the p27 viral antigen (Gelmetti *et al.*, 2000; Benavides *et al.*, 2006b).

Serological Tests

Serum samples were obtained from all of the sheep to evaluate the presence of antibodies against MVV using a commercial test (Elitest®, Hyphen BioMed, Neuville-Oise, France) in accordance with the manufacturer's instructions. Enzyme linked immunosorbent assay (ELISA) results were reported as positive or negative on the basis of the cut-off value calculated as per the manufacturer's instructions (absorbance at 450 nm).

Polymerase Chain Reaction

Genomic DNA from the 10 animals that were not tested by IHC for the p27 protein of MVV was extracted from peripheral blood mononuclear cells and tissue samples using the QIAamp® DNA Blood Mini Kit (Qiagen, Madrid, Spain). Amplification of LTR and *gag* regions from animals with nervous lesions was performed as described by Reina *et al.* (2006).

Results

All of the animals had characteristic lesions of MV, although there were clear differences between individual cases. The 10 animals tested for MVV by PCR were positive and all 34 animals were seropositive. The description and characterization of nervous lesions were based on the location, extent of injury and predominant cell type (Fig. 1) and the results of immunohistochemical studies are summarized in Table 2. The 24 sheep tested showed immunohistochemical expression of the p27 MVV antigen. This positive signal appeared as fine granular deposits in the cytoplasm of macrophage-like cells. Although the assessment was subjective, positive p27 labelling was clearly more abundant in histiocytic lesions compared with lymphocytic lesions (see below).

Lymphocytic Lesions

Lymphocytic lesions were characterized by non-purulent encephalitis with infiltrates dominated by lymphocytes. Scattered vacuoles of demyelination were occasionally present. This type of lesion was further subdivided into two subtypes.

Lymphocytic Type A. This lesion was found in the choroid plexus and meninges. There was no apparent change or only mild lesions in the adjacent neuroparenchyma. Hyperplasia of the lymphoid tissue, with occasional aggregates or lymphoid follicles, was observed (Fig. 1). These lesions had a clear predominance of T cells ($CD3^+$) in the infiltrates of the choroid plexus and meninges (Fig. 2). A high proportion of $CD4^+$ versus $CD8^+$ T cells was observed where there was mild choroiditis and meningitis, but the proportions became similar in lesions with the presence of lymphoid aggregates or follicles. The lymphoid aggregates appeared as a follicular arrangement with numerous $CD4^+$ T cells located in the active germinal centres and $CD8^+$ lymphocytes both in the periphery and in the centre of follicles, adjacent to macrophages. B cells were rarely detected, except in the areas of well-developed lymphoid follicles where the germinal centres were composed mainly of B cells. In the case of mild lesions in the adjacent neuroparenchyma, mononuclear cells around blood vessels were mainly $CD4^+$ T cells, together with $CD8^+$ lymphocytes in multilayered perivascular cuffs. $CD8^+$ cells also appeared adjacent to the periventricular neuroparenchyma and in small foci of gliosis. Few lymphocytes expressing the γδ form of the T-cell receptor (TCR) or macrophages were present.

Lymphocytic Type B. This lesion was located in the neuroparenchyma with the choroid plexus and meninges only mildly infiltrated, and mainly by lymphocytes. A mononuclear cell infiltrate with a clear predominance of lymphocytes with macrophages interspersed between them, and foci of gliosis as well as perivascular cuffing, were the most significant changes (Fig. 1). T cells ($CD3^+$) were the most abundant population. Both the meninges and the choroid plexus were lightly infiltrated by T cells and these were mainly $CD8^+$. An extensive infiltration of T cells, particularly at the edges of the lesion, was observed in the neuroparenchyma (Fig. 3). Most of the T cells were $CD8^+$ in the foci of gliosis foci and perivascular cuffs, and there were fewer $CD4^+$ cells. $CD4^+$ lymphocytes were in similar number to $CD8^+$ cells in multilayered perivascular cuffs (Fig. 4). Macrophages were present in moderate number and were mainly located in the neuroparenchyma of the central area of the lesion, or at the edges of the lesion around slightly damaged blood vessels forming mono- or bilayers (Fig. 3). Few γδ TCR-bearing T cells or B lymphocytes were detected,

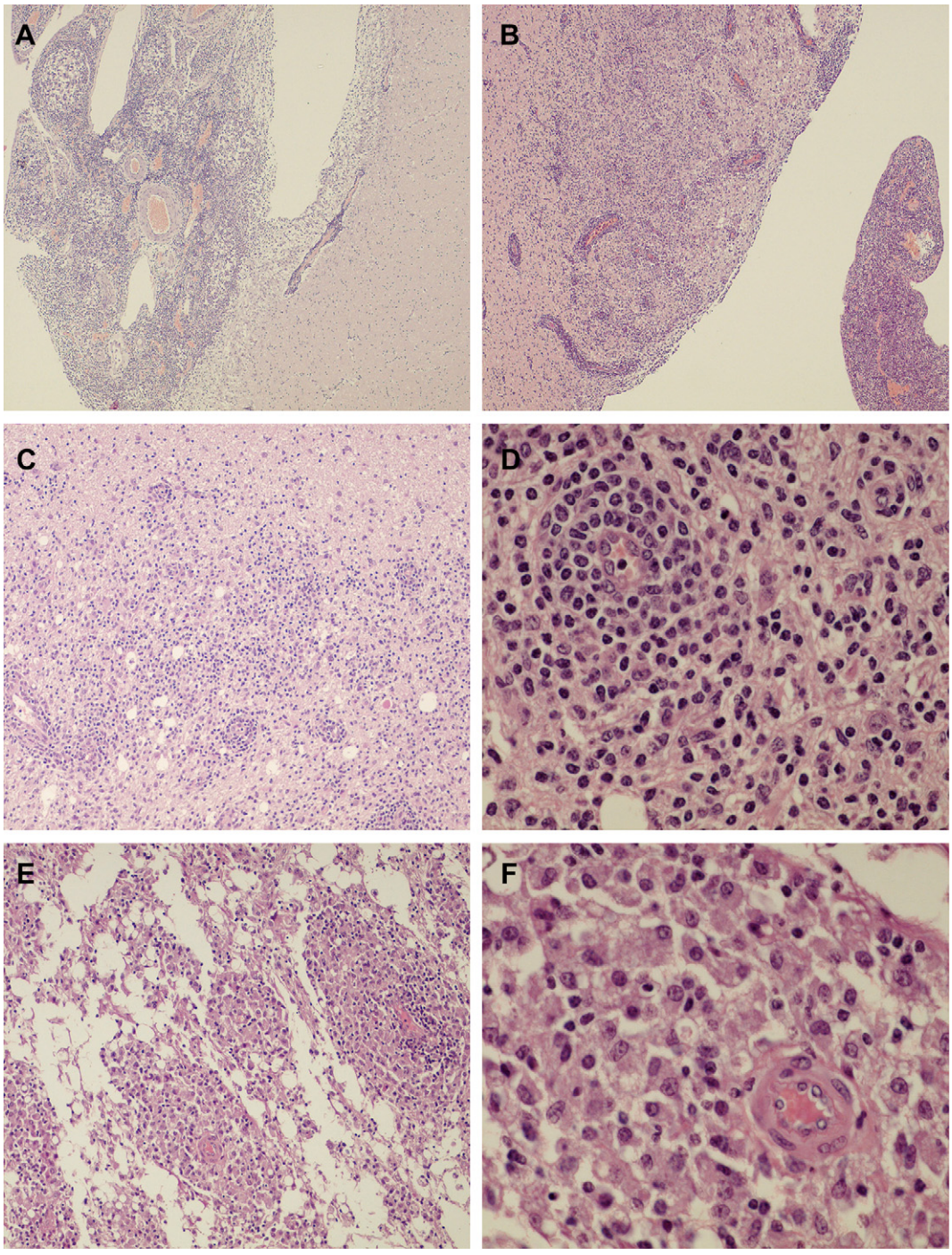

Fig. 1. Nervous lesions in natural cases of ovine MV. (A) Lymphocytic type A. Non-purulent choroiditis composed mainly of lymphocytes with no apparent lesions or mild lesions in the adjacent neuroparenchyma. Hyperplasia of the lymphoid tissue, which may develop aggregates or lymphoid follicles, is observed. (B) Histiocytic type A. The main lesion is located in the choroid plexus and meninges and the adjacent neuroparenchyma has moderate–severe damage. Numerous macrophages are present in the periventricular area and vacuoles consistent with demyelination and perivascular cuffs are observed commonly. (C) Lymphocytic type B. Non-purulent encephalitis characterized by a mononuclear cell infiltrate with a predominance of lymphocytes with macrophages interspersed between them. There are also foci of gliosis foci and perivascular cuffs. (D) Higher magnification of (C). (E) Histiocytic type B. The neuroparenchyma is the most affected area with extensive areas of malacia and a non-purulent infiltrate with predominance of large and foamy macrophages (gitter cells). (F) Higher magnification of (E). HE. (A–B) ×40, (C) ×100, (D) ×400, (E) ×100 and (F) ×400.

Table 2
Summary of IHC results

Cell types	*Lymphocytic type A*	*Lymphocytic type B*	*Histiocytic type A*	*Histiocytic type B*
T cell	+ +	+ + +	+ +	+ +
Macrophages	+	+	+ + +	+ + +
$CD8^+$ T cell	+	+ + +	+ +	+ +
$CD4^+$ T cell	+ +	+ +	+	+
B cell	±	±	+ +	+ +
γδ T cell	+	±	±	±

±, few scattered positive cells; +, moderate number of positive cells; + +, large number of positive cells; + + +, predominant cell type.

with γδ T cells being scattered in the parenchyma and B cells always close to the vessels (Fig. 5).

Histiocytic Type

This pattern was characterized by severe lesions with a predominance of macrophages with foamy cytoplasm and extensive areas of malacia. Two subtypes were recognized.

Histiocytic Type A. The main lesion was located in the choroid plexus and meninges, with moderate to severe change of the adjacent neuroparenchyma. Although infiltration of the choroid plexus was predominantly lymphocytic, a greater number of macrophages were present in contrast to the type A lymphocytic lesion. A clear predominance of macrophages in the periventricular area, with vacuolation representing demyelination and perivascular cuffing, was often observed (Fig. 1). The choroid plexus infiltrate was composed mainly of T and B cells, but with a high proportion of macrophages. Numerous B cells were observed in the lymphoid follicles and in the periventricular parenchyma. Macrophages with expanded foamy cytoplasm were clearly the predominant cells in the adjacent damaged neuroparenchyma, where they appeared together with some T cells (Fig. 2). The T cells were mostly $CD8^+$ and were located in foci of gliosis and in perivascular cuffs. $CD4^+$ lymphocytes appeared less frequently and were located mainly around blood vessels. Many macrophages and lymphocytes were detected around multilayered perivascular cuffs, while in vessels with mild damage at the edge of the lesions, only a single layer of macrophages adjacent to the endothelium was observed. There were also numerous B cells, mainly located adjacent to vessels in the periphery of the lesion. In this case, γδ T cells were also scattered among the other cell types.

Histiocytic Type B. In this lesion the neuroparenchyma was the most affected area and the most severe lesions had this pattern. A non-purulent infiltrate with predominance of large macrophages as compared with lymphocytes was observed. Clusters of 'gitter cells' were observed near blood vessels, where they formed large perivascular cuffs. This change was usually accompanied by intense focal to coalescing areas of vacuolation, forming extensive areas of malacia (Fig. 1). In this type of lesion, the predominance of macrophages with foamy cytoplasm and demyelination in the neuroparenchyma was the main feature (Fig. 3). T cells were mainly ranged along the edges of the lesion (Fig. 3) and the proportion of $CD8^+$ T cells was greater than that of $CD4^+$ cells. Perivascular cuffs were composed of several layers, with the external layers consisting mainly of macrophages with $CD8^+$ and $CD4^+$ T lymphocytes located closer to the endothelium (Fig. 4). Mildly

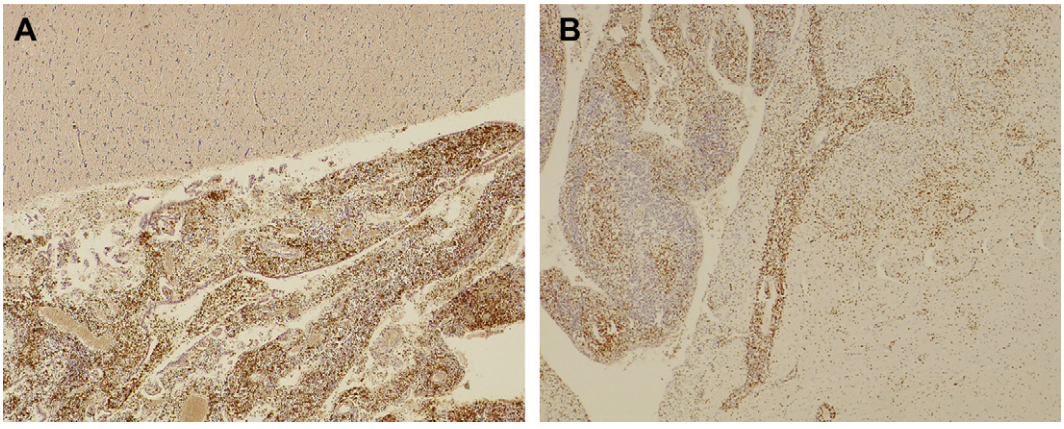

Fig. 2. Phenotypic characterization of cell populations present in each histological type of lesion. (A) Lymphocytic type A. There is a predominance of T cells in the infiltrate of the choroid plexus and meninges. Occasional T cells are located in the adjacent neuroparenchyma. (B) Histiocytic type A. Small number of T cells in the choroid plexus compared with lymphocytic type A. Numerous T cells are present in the adjacent neuroparenchyma. IHC. ×40.

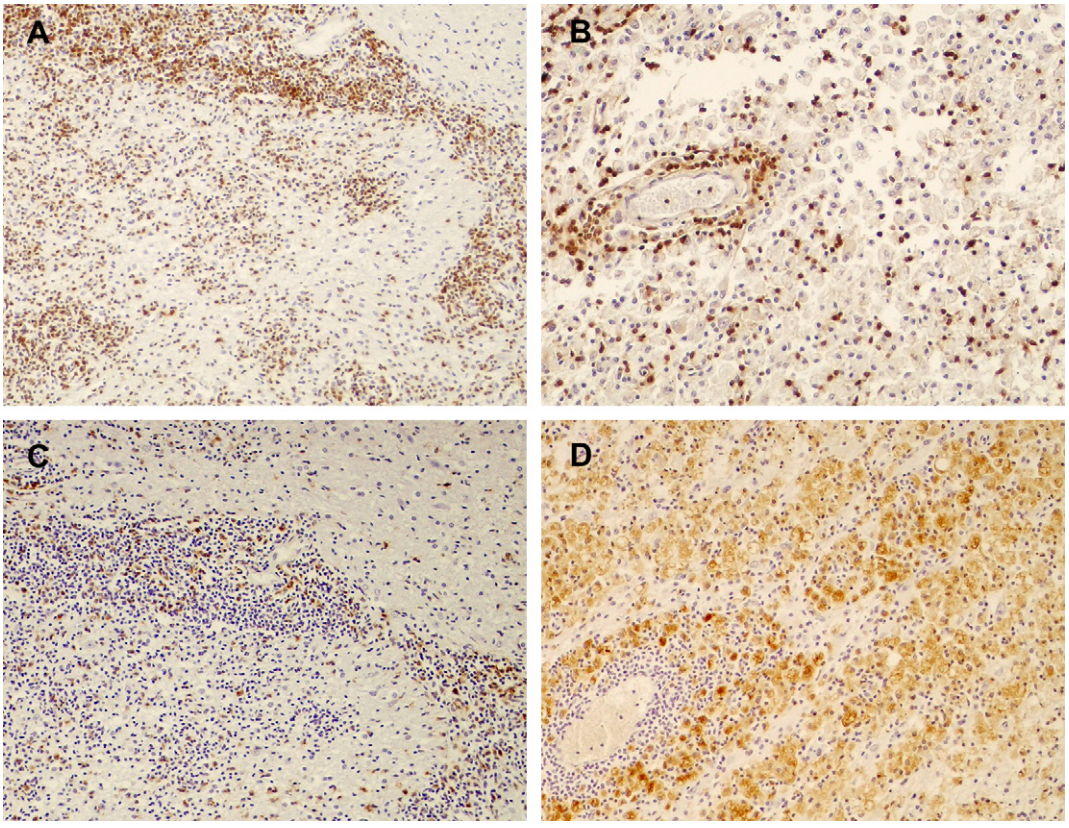

Fig. 3. Phenotypic characterization of cell populations present in each histological type of lesion. (A) Lymphocytic type. Extensive labelling of $CD3^+$ T cells in perivascular cuffs and the surrounding parenchyma. (B) Histiocytic type. Fewer $CD3^+$ T cells are present compared with the lymphocytic type. (C) Lymphocytic type. $CDD68^+$ macrophages in moderate number are located in the central area of the lesion and around blood vessels. (D) Histiocytic type. Predominance of $CD68^+$ macrophages with foamy cytoplasm. IHC. (A) ×100, (B) ×150, (C) ×100 and (D) ×150.

damaged vessels were completely surrounded by macrophages. In this type of lesion, a considerable number of B lymphocytes were found close to the endothelium, especially at the edges of the lesion, and some γδ T cells were detected (Fig. 5).

Discussion

The nervous form of MV has been studied by several authors who have described the lesions associated with the disease (Sigurdsson *et al.*, 1957; Sigurdsson and Palsson, 1958; Benavides *et al.*, 2006a, 2009). These studies have been taken as a reference for the histopathological study and diagnosis of the disease. The characterization by IHC of the cellular population of each lesion type is of value for further understanding the pathogenesis of the disease, due to the important role of cell-mediated immunity in determining the severity of the CNS lesions (Torsteinsdottir *et al.*, 1992). Likewise, the balance between cell populations and their interactions appears to determine the evolution of viral infection (Dorries, 2001) and this may be of importance in MV, given that the significant differences found in this study between the lesions of the individual animals. Thus, based on these observations, one dominant lesion pattern can be established for each affected animal.

In this context, an important finding of the present study was that the lymphocytic type A lesion, affecting chiefly the choroid plexus and meninges with mild lesions in the adjacent neuroparenchyma, had a predominance of $CD4^+$ T cells. The description of this lesion type is similar to lesions described previously in the early stages of CNS infection by other lentiviruses [e.g. CAEV, feline immunodeficiency virus (FIV) and human immunodeficiency virus (HIV)-1], where up to 70% of lymphocytes and 10% of macrophages/microglia are observed around the perivascular cuffs. In such lesions, $CD4^+$ T cells are more numerous in perivascular cuffs, while the $CD8^+$ T-cell infiltrate is mainly distributed in the neuroparenchyma (Torsteinsdottir *et al.*, 1992). Thus, this lesion type could be related to early stages of the disease or a different mechanism of resistance to the progression of the infection. In addition, neuroinvasion of MVV may be

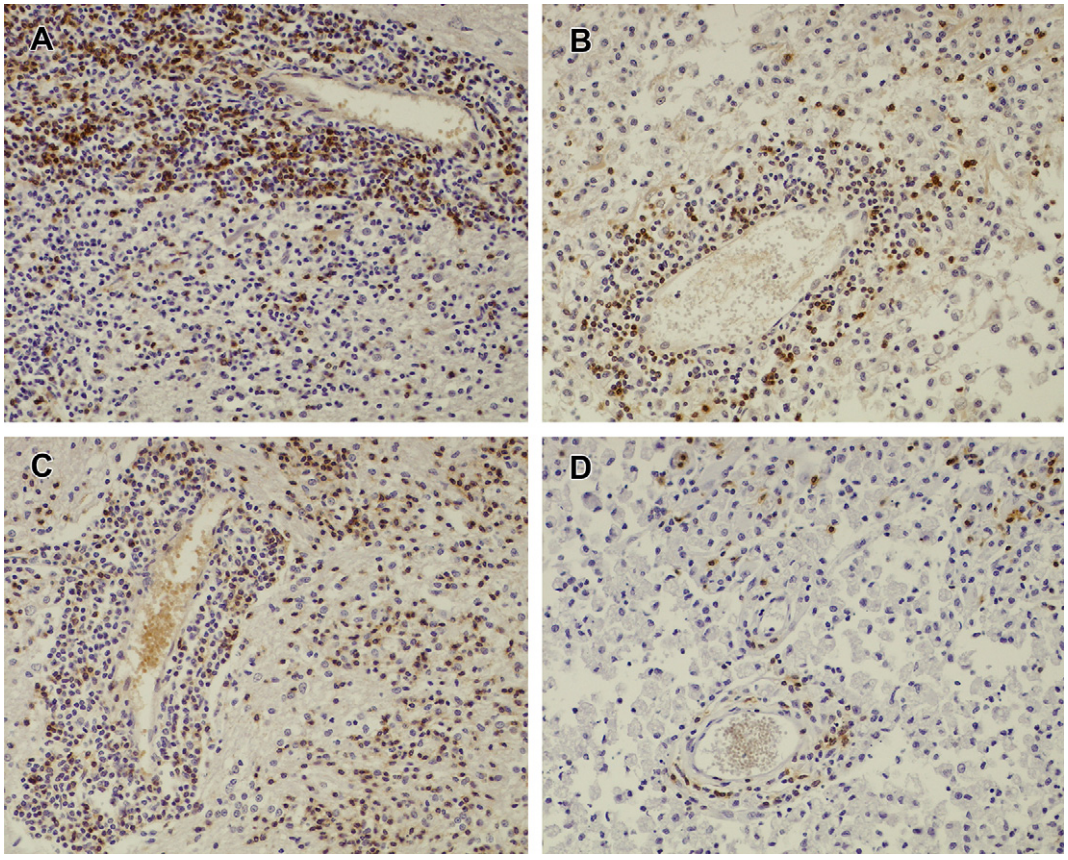

Fig. 4. Phenotypic characterization of cell populations present in each histological type of lesion. (A) Lymphocytic type. $CD4^+$ T cells in multilayered perivascular cuffs and in low number in the neuroparenchyma. (B) Histiocytic type. $CD4^+$ T cells closer to the endothelium. (C) Lymphocytic type. $CD8^+$ T cells in perivascular cuffs and infiltrating the neuroparenchyma. (D) Histiocytic type. Fewer $CD8^+$ T cells are present than in lymphocytic lesions. IHC. ×200.

initiated by activated peripheral T cells that migrate to the CNS to facilitate recruitment of monocytes (Chebloune *et al*., 1998). Moreover, an experimental study showed that immunosuppression with anti-lymphocyte serum prevented the cellular proliferative response and the development of lesions (Panitch *et al*., 1976). Because of these observations, the presence of lymphocytes in mild lesions and in layers around vessels (lymphocytic types) suggests an initial recruitment of lymphocytes into the CNS and might represent the first response to viral infection. Initial mammary and arthritic (CAE) lesions have also been studied in experimentally-infected animals, showing an accumulation of activated $CD4^+$ and $CD8^+$ T cells in tissues, mainly around blood vessels, with only a few B cells (Cordier *et al*., 1992; Von Bodungen *et al*., 1998). This pattern is similar to the lymphocytic types of lesions described here for the CNS.

In the present study, type B lymphocytic lesions were relatively severe and had more $CD8^+$ T cells infiltrating the neuroparenchyma than other patterns. Although $CD8^+$ T cells are thought to be important in controlling viral replication, their role in the CNS is still unknown. Increased tissue damage due to the cytotoxic action of these cells or to an exacerbated pro-inflammatory cytokine secretion has been suggested to play a role in disease pathogenesis (Torsteinsdottir *et al*., 2007). Following viral infection of the CNS, a Th-1 type immune response stimulates the recruitment of $CD8^+$ T cells, which in turn results in marked up-regulation and secretion of chemotactic substances and, consequently, an increasing number of inflammatory cells are attracted to the site of virus infection (Dorries, 2001). In this context, this lesion type may represent a transitional step towards the more severe lesions or another mechanism of immune response where $CD8^+$ T cells play an important role.

In contrast, both types of histiocytic lesion involved severe changes to the neuroparenchyma and may represent an advanced stage with the recruitment of monocytes/macrophages due to an exaggerated, but probably ineffective, immune response. In this regard, in the late stages of other lentiviral infections [e.g. simian immunodeficiency virus (SIV), HIV-1] monocytes/macrophages comprise 90—95% of the cellular infiltrate, with the remainder of the cellular

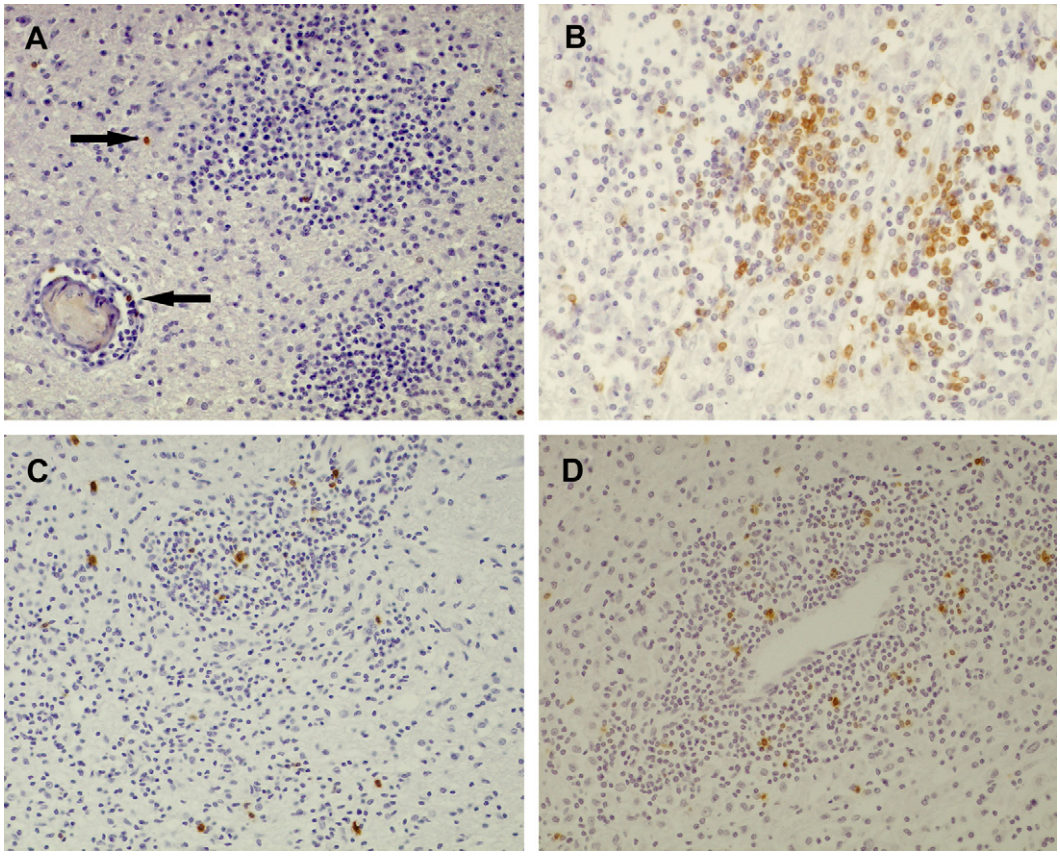

Fig. 5. Phenotypic characterization of cell populations present in each histological type of lesion. (A) Lymphocytic type. Arrows indicate occasional CD79a$^+$ B cells. (B) Histiocytic type. Extensive B-cell labelling in the lesion. (C) Lymphocytic type. Labelling for T cells expressing the γδ TCR. (D) Histiocytic type. A small number of γδ T cells are scattered in the neuroparenchyma and closer to the endothelium. IHC. ×200.

population being CD8$^+$ T cells (Lackner *et al.*, 1991; Kim *et al.*, 2004). Moreover, CAE arthritic lesions of both naturally- and experimentally-infected animals with advanced lesions have a significant accumulation of macrophages and B cells in the inflammatory tissue (Von Bodungen *et al.*, 1998). The increase in B cells suggests that the immune response to infection may have shifted towards a Th2 response, losing effectiveness, as has been previously described in arthritic lesions of goats infected with CAEV (Wilkerson *et al.*, 1995). Similarly, evidence for an ineffective T-cell response is described *in vitro* in severely affected animals in terms of antigen recall responses due to impaired presentation related to deficiency in expression of co-stimulatory molecules (Reina *et al.*, 2007). All of the younger sheep ($n = 7$) had histiocytic lesions, suggesting that these lesions may have a relatively rapid development and consequently represent an ineffective immune response.

The amount of viral antigen was greater in the histiocytic types of lesion. This may reflect the large number of macrophages in this type of lesion or indicate a lesion pattern with low resistance to viral replication. However, IHC for detection of MVV was not carried out on enough animals to consider this feature as significant.

Differences in the location of the main lesion (i.e. choroid plexus and meninges for type A or the neuroparenchyma for type B lesions) might be explained by the route of entry of virus to the CNS, via the blood–brain barrier (BBB) or via the blood–cerebrospinal fluid barrier. Viral migration through the BBB to reach the neuroparenchyma could be the first step in developing encephalitis (type B), while in the case of migration through the choroidal or meningeal endothelial barrier, choroiditis/meningitis would be the expected lesions (type A). Both routes of entry of MVV to the CNS have been proposed (Haase *et al.*, 1977; Peluso *et al.*, 1985; Georgsson *et al.*, 1989; Georgsson, 1994; Drevets and Leenen, 2000; Benavides *et al.*, 2009).

The pathogenesis of the disease has been hypothesized to involve damage to the BBB due to the entry of MVV, with resultant release of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α and the overexpression of endothelial adhesion

molecules, leading to changes in vascular permeability and the increased migration of inflammatory cells to the CNS (Craig *et al*., 1997). This step could be represented by the lymphocytic types of our classification. Subsequently, continuous immune activation may induce the differentiation and attraction of B cells and macrophages with an associated increase in the secretion of cytokines, thereby establishing a 'vicious circle' (Ebrahimi *et al*., 2000; Torsteinsdottir *et al*., 2007). According to this hypothesis, the histiocytic lesions would represent advanced stages with severe clinical consequences related to the infiltration of macrophages and B cells. Numerous viral strategies to escape immune-mediated cellular responses have been described. These may result in life-long persistence of virus in the brain, therefore the presence of chronically-infected animals could be due to such control of viral replication and may be represented by the lymphocytic types of lesion.

In conclusion, these lesion types could be related to different stages or different mechanisms of resistance to the disease. The lymphocytic lesions appear to be initial or latent phases of slow progression in which the animal has some form of natural resistance to the infection. The histiocytic pattern is more severe and extensive and may be due to an individual poor immune response or a greater virulence of the viral strain. This detailed description of nervous lesions represents an improvement in the study of the individual response to MVV infection and the evolution of the disease and its diagnosis. The present work provides a basis for future studies of MV in particular of the individual immune response controlling progression of the infection and in the evaluation of vaccines, treatments or individual resistance to the disease.

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SHORT COMMUNICATION

Perivascular inflammatory cells in ovine Visna/maedi encephalitis and their possible role in virus infection and lesion progression

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Abstract We examined the distribution in the perivascular spaces of Visna/maedi antigen, T cells (CD3+, CD4+ and CD8+), B cells and macrophages by immunohistochemistry in 22 natural cases of Visna/maedi encephalitis. Sheep showed *lymphocytic* or *histiocytic* lesions. In mild lymphocytic lesions, the viral antigen was detected in perivascular cuffs where CD8+ T cells predominated, but in severe lymphocytic lesions, sparse antigen was identified, and CD8+/CD4+ T cells appeared in a similar proportion in multilayer perivascular sleeves. In histiocytic lesions, vessels were surrounded by macrophages with abundant viral antigen, with CD8+/CD4+ T cells and B cells in the periphery. These results could reflect different stages of virus neuroinvasion and clarify the neuropathogenesis of Visna/maedi encephalitis.

Keywords Maedi · Visna · Sheep · Encephalitis · Neuropathology · Immunology

Introduction

Visna/maedi virus (VMV) is a lentivirus of the *Retroviridae* family which is related to human immunodeficiency virus (HIV-1) (Thormar 2005) and causes a slow, progressive multi-systemic disease in sheep. Visna/maedi disease (VM) is mainly characterised by chronic inflammation of the lungs, central nervous system (CNS), mammary glands and joints (Cutlip et al. 1988; Dawson 1987). The disease is most commonly presented in respiratory and mammary forms, while the neurological form has often been sporadic (Benavides et al. 2006a, 2009; Lujan et al. 1991; Sigurdsson et al. 1957). The main histologic changes are interstitial inflammation of the lungs and mammary glands with proliferation of lymphoid tissue, as well as non-purulent encephalitis and demyelination of the CNS, including the spinal cord (Benavides et al. 2009; Georgsson et al. 1976; Lujan et al. 1991; Sigurdsson et al. 1957).

In the region of Castilla y León (Spain), VM is considered a widespread disease, with a prevalence estimated at 77 %, especially in the Assaf dairy flocks subjected to an intensive farming setup (Leginagoikoa et al. 2006). In this region, a previous study showed that a proportion as high as 11. 2 % of the sheep showing nervous clinical signs were suffering from the VM (Benavides et al. 2009, 2006c; Gómez et al. 1999). Nervous clinical signs often include progressive ataxia, limb weakness and paresis, particularly in the hind limbs, usually leading to total paralysis and recumbency although the animal remains alert (Benavides et al. 2006c; Christodoulopoulos 2006; Polledo et al. 2011; Sigurdsson et al. 1957). The primary lesion in the brain or spinal cord is a non-suppurative encephalitis predominately periventricular and paraventricular, accompanied or not by non-suppurative choroiditis and meningitis (Benavides et al. 2009; Polledo et al. 2011; Sigurdsson et al. 1957).

The immune response against VMV seems to play a major role in the pathogenetic mechanism; thus, an imbalance in the immune response, whether excessive or deficient, would result in lesion development (Blacklaws 2012; Polledo et al. 2011; Torsteinsdottir et al. 2007, 1992). Once in the host, the main targets of VMV are monocytes/macrophages and dendritic cells, which carry the viral DNA in blood with minimum transcription until the monocytes mature into macrophages in the tissue of affected organs

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("Trojan horse" mechanism) (Peluso et al. 1985). It appears that the entry of VMV into the CNS may damage the blood–brain barrier (BBB), leading to changes in vascular permeability and increasing migration of inflammatory cells (lymphocytes and monocytes) to the CNS with secretion of cytokines, creating a vicious circle (Craig et al. 1997; Ebrahimi et al. 2000; Georgsson 1994; Georgsson et al. 1976; Torsteinsdottir et al. 2007). However, this pathological mechanism is not yet fully understood.

A previous study has shown that one sheep with VM nervous lesion showed changes characterised by a diffuse inflammatory infiltrate in the neuroparenchyma with a CD4/CD8 ratio of 0.8 and in the perivascular spaces composed of lymphocytes with a CD4+/CD8+ ratio of 1/3 and 10 % monocytes (Torsteinsdottir et al. 1992), similar to other lentiviruses (CAEV, FIV and HIV-1) (Georgsson 1994). Recently, two main patterns of lesion have been described in relation to each animal: a *lymphocytic type*, where areas of mild–moderate injury characterised by a clear predominance of T cells, mainly CD8+ lymphocytes, are observed, and a *histiocytic type*, characterised by more severe lesions with clear predominance of macrophages, many of them with foamy cytoplasm, mixed with B cells, that is usually accompanied by intense vacuolation foci that coalesce, forming extensive malacic areas.

These lesion patterns could be related to different stages or mechanisms of resistance to the disease. Thus, the lymphocytic lesions could represent some sort of natural resistance to the infection where initial or latent phases are included, and the histiocytic pattern may be the result of an individual poor immune response or greater virulence of the viral strain (Polledo et al. 2011). In this study, we investigated the immunophenotype and distribution of the inflammatory cells specifically within the vascular spaces in relation to the viral antigen and the different patterns of lesion, for the purpose of studying the role of the perivascular spaces in virus neuroinvasion and in the development of the neuroparenchymal lesions (histiocytic or lymphocytic lesions).

Brain tissue samples from natural cases of Visna/maedi encephalitis in 22 adult sheep (over 2 years old) of the Spanish Assaf breed were examined. All of them were naturally infected cases which had been submitted to the Pathology Diagnosis Service of the Veterinary School of León with nervous clinical signs and had been diagnosed as VM infected. CNS tissue samples were obtained systematically, taking different sections: cortex, diencephalon, corpus callosum, hippocampus, midbrain, cerebellar cortex, pons and cerebellar peduncles, medulla oblongata, and cervical, thoracic and lumbar spinal cord. Sample tissues were fixed in 10 % buffered formalin, stained with haematoxylin–eosin and examined by light microscopy. Samples from these same locations were fixed in zinc salt fixative (0.5 % zinc chloride, 0.5 % zinc acetate in 0.05 % Tris buffer, 0.1 M calcium acetate, pH=7.4) and tested using immunohistochemical techniques (IHC) using serial tissue sections. These serial tissue sections were stained with primary antibodies raised against the p27 VM viral antigen (Gelmetti et al. 2000), as previously described (Benavides et al. 2006b), and T cells (CD3+, CD4+ and CD8+), B cells (CD79 αcy) and macrophages (CD68), using previously described IHC staining procedures (Polledo et al. 2011). Samples from a sheep without characteristic lesions of VM which showed negative results to PCR procedures and serology were used as negative control. A semiquantitative analysis of the presence of the different cell immunophenotypes in perivascular cuffs associated with the inflammatory response to VM antigen was carried out.

Serum samples were obtained from the 22 sheep to evaluate the presence of antibodies against VMV using a commercial test (Elitest®, Hyphen BioMed, Neuville/Oise, France), following the manufacturer's instructions. ELISA results were reported as positive or negative on the basis of the cutoff value calculated following the manufacturer's instructions (absorbance, 450 nm).

Histological examination revealed that all the 22 studied sheep presented characteristic CNS lesions of VM encephalitis, 9 sheep showed lymphocytic lesions and 13 sheep showed histiocytic lesions. Five sheep that showed mild nervous signs (lethargy, tremors, incoordination or mild ataxia of the hind limbs) presented non-suppurative lymphocytic encephalitis consisting only in perivascular cuffs spread throughout the neuroparenchyma, characterised by the arrangement of round cells in mono- or multiple layers around blood vessels with no or sparse infiltration of the neuropil, named *mild lymphocytic lesions* in this study. Another four sheep with more severe nervous clinical signs (progressive ataxia and recumbency) showed these similar mono- and multilayer perivascular cuffs accompanied by a lymphocytic infiltrate in the neuroparenchyma, and these were considered *severe lymphocytic lesions*. Thirteen animals showed histiocytic lesions with extensive areas of malacia and a non-suppurative histiocytic infiltrate with evident predominance of large and foamy macrophages. These 13 sheep with histiocytic encephalitis also showed severe nervous signs of progressive ataxia and recumbency, but five of them with more than 2 weeks of recumbency. The 22 sheep yielded positive results to the serological tests.

VMV antigen was detected in the brain tissue sections of all the animals in this study, while the control sections were negative. The positive signal was found predominantly in the cytoplasm of the macrophages/microglia located in the CNS lesions. The result of the analysis of the distribution of the viral antigen and the different perivascular cell types associated with the inflammatory response to VMV infection is detailed in Table 1. These results showed clear

Table 1 Visna/maedi antigen distribution and cell immunophenotypes forming the perivascular cuff layers

	Mild lesion		Lymphocytic lesion		Histiocytic lesion	
PC	Monolayer	Multilayer	Monolayer	Multilayer	Monolayer	Multilayer
VM antigen	++	++	S	S	+	+
T cell CD3+	+++	+++	+++	+++	+	++
T cell CD8+	+++	++	+++	++	+	++
T cell CD4+	+	++	+	++	+	++
Macrophage	+	+	+	+	+++	++
B cells	S	S	S	S	+	++

Results of semiquantitative analysis of viral antigen and the cell immunophenotypes in perivascular spaces of mild, lymphocytic and histiocytic lesions, scored as s, sporadic; +, few; ++, moderate, and +++, many

PC Perivascular cuffs formed by cellular mono- or multiple layers

differences in the phenotypical cellular composition in perivascular spaces in various types of lesion and in the distribution of the VM antigen.

In mild lymphocytic lesions, where perivascular cuffs seemed to be the only damage, with scarce inflammatory response in the neuropil, VM antigen immunoreactivity was detected in these perivascular sleeves. The antigen-positive signal was found in the cytoplasm of macrophage-like cells close to the endothelium, and mixed between the cellular sleeves, but it was hardly detected in the neuroparenchyma (Fig. 1). Interestingly, this finding has been observed in this type of lesion alone, so this antigen immunolabelling could correspond to infected perivascular macrophages, or circulating infected monocytes that differentiate into macrophages entering the CNS by this route and allowing viral replication. These observations would confirm the previously proposed models of VMV neuroinvasion, based on the "Trojan horse" pathological mechanism (Georgsson et al. 1989; Peluso et al. 1985). In this way, in other lentivirus infections such as simian immunodeficiency virus (SIV) and HIV-1, it has been demonstrated that perivascular macrophages are the primary cells productively infected by the virus, resulting in a disruption of the BBB and allowing greater entry of infected cells into the CNS (Kim et al. 2003; Persidsky, 1999; Strazza et al. 2011; Williams et al. 2001b), as could initially occur in VM encephalitis. Thus, most cells (over 90 %) which made up these perivascular sleeves were T cells mixed with scattered macrophages, often close to the endothelium. Specifically, the CD8+ T cell subpopulation clearly predominated over CD4+ T lymphocytes in the mono/bilayer perivascular cuffs, but larger multilayer perivascular cuffs showed these cells in similar proportions of 50 %. B cells were only sporadically observed. Thus, it is possible that in mild VM encephalitis, the CD8+ lymphocytes located around blood vessels may be cytotoxic effector cells when they encounter cells presenting viral antigen. Likewise, in SIV encephalitis, it has been reported that CD8+ lymphocytes located angiocentrically appear to control the accumulation of infected macrophages in the CNS in an antigenic-specific manner, also with little accumulation of CD4+ lymphocytes (Freel et al. 2011; Kim et al. 2004). Specifically, this feature has been demonstrated in SIV infection when the viral load was increased, and progression of the disease was accelerated, in animals whose CD8+ T cells were depleted (Schmitz et al. 1999; Williams et al. 2001a). However, it has previously been suggested that the

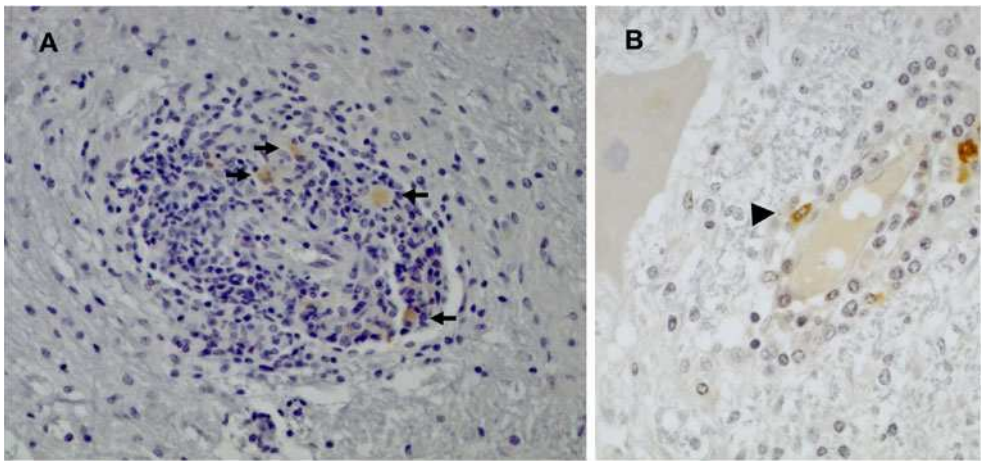

Fig. 1 Cerebellar peduncles (**a**) and mild lymphocytic lesion (**b**) of VM encephalitis essentially composed of perivascular cuffs with positive antigen signal in macrophage-like cells mixed between the cellular sleeves (*arrows*). Note that in **b** the antigen is located close to the endothelium (*arrowhead*). Anti-p27 VM antigen IHC. **a** ×150, **b** ×250

cellular immune response of CD8+ together with CD4+ T lymphocytes in inflammatory lesions of VM may be directed not only against the virus but also against self-antigen (Blacklaws 2012; Torsteinsdottir et al. 2007, 1992), so the presence of CD4+ T cells together with CD8+ T cells in multilayer perivascular cuffs detected in this study could be representing a stage of lesion progression to more severe lymphocytic lesions.

In CNS lesions with a more severe lymphocytic infiltrate of the neuroparenchyma (*severe lymphocytic lesions*) with CD8+ lymphocyte predominance, the presence of antigenic positive signal was very sparse in the perivascular spaces and the neuropil (Fig. 2). Cell immunophenotypes which formed both mono/bilayer and multilayer perivascular cuffs were very similar to the ones previously described in mild lymphocytic lesions, but with a greater abundance of multilayer perivascular sleeves. In severe lymphocytic lesions, despite the possible role of the CD8+ T cells in the control of viral replication, this vascular lymphocytic inflammatory response could also cause tissue damage due to dysregulation of the immune response. In this way, in SIV encephalitis lesions, it has also been reported that a large number of activated CD8+ T lymphocytes accumulate abnormally in the brain, resulting in increased concentrations of cytokines to pathological levels (Marcondes et al. 2001). Thus, the presence of this lymphocytic infiltrate may be essential in controlling the infection, although it may also contribute to the progression of the VM lesion. In these perivascular spaces, the number of B cells was very low, so this feature indicates that the humoral immune response would be minimal in this type of lesion, with cellular immunity playing the major role.

In particular, examination of lesions in the CNS with histiocytic infiltration revealed a high number of VMV-positive cells in the central areas of the lesion, mixed with malacia (Fig. 3), but this antigen-positive signal was rarely detected immediately adjacent to the endothelium or in perivascular cuffs. In these histiocytic infiltrates, macrophages with a clear cytoplasm ("gitter cells") clearly predominated over other cells such as CD8+ and CD4+ T cells and B cells. These observations would support the hypothesis of development of VM encephalitis after neuroinvasion of the CNS. Thus, once in the tissue, infected monocytes mature and allow viral replication and recruitment of more infected cells, based on an immune activation in response to viral antigen that also causes inflammatory infiltration and damage of the CNS tissue (Blacklaws 2012; Torsteinsdottir et al. 2007). This pathological model would explain the abundance of macrophages and viral antigen seen in the parenchyma in the histiocytic lesion. This way, a slow rate of VM neutralization by antibodies relative to the rate of virus adsorption to the cell surface has been suggested as a possible mechanism whereby the virus can spread from cell to cell in the presence of neutralizing antibodies with no free virus release (Thormar 2005). This feature could explain the persistence and replication of virus in the neuroparenchyma of the histiocytic lesion in the face of an active immune response. Likewise, the viral induction of apoptosis has been proposed as the major mechanism of cell death occurring during MVV infection (Duval et al. 2002a), and this mechanism is considered to promote cell-to-cell spreading, virus release and stimulation of the immune response (Duval et al. 2002b).

The IHC study of vascular spaces in the areas with abundant malacia of the histiocytic lesions, often central areas, showed that some blood vessels were completely surrounded by the macrophage infiltration, with few B cells and T cells (mainly CD8+ lymphocytes, with CD4+ in lower proportions) adjacent to the endothelium that

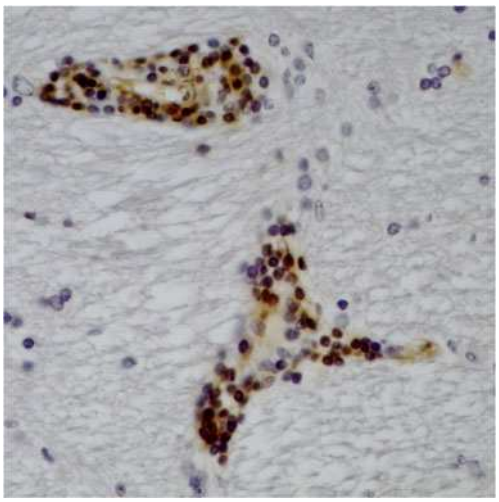

Fig. 2 Cerebellar peduncles, mononuclear cells around blood vessels in lymphocytic lesions of VM encephalitis, with clear predominance of CD8+ T cell immunoreactivity. Anti-CD8+ T cell IHC. ×250

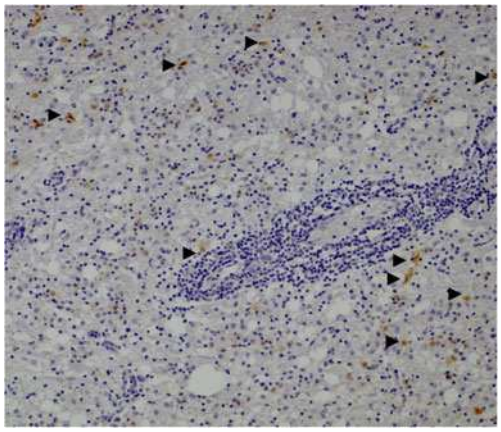

Fig. 3 Cerebellar peduncles, histiocytic inflammatory demyelination lesion of VM encephalitis. Antigen-positive signal (*arrowheads*) located in the malacic area. Anti-p27 VM antigen IHC ×100

was occasionally disrupted. In the periphery of the lesion, where multilayer perivascular cuffs were often located, the layers close to the endothelium were mainly formed by T cells (CD8+ and CD4+, in similar proportions) with around 20 % of B cells, and macrophages behind these layers. This lymphocytic perivascular cuffs may provide a cellular immune response similar to the one described for lymphocytic lesions in response to the spread of the lesion and the consequent endothelial damage. In addition, the observed CD4+ T cells together with B cells tightly packed in these perivascular cuffs may produce an effective utilisation of CD4+ lymphocytic cytokines by B cells, resulting in strong antibody production (Esiri and Gay, 1990) that may be directed not only against the virus, but also against self-antigens although this is still not clear (Panitch et al. 1976; Torsteinsdottir et al. 2007). Thus, in these histiocytic lesions, a non-effective humoral immune response may be involved in the development of tissue damage.

To summarize, this study reinforces and clarifies the prior model of development of VM encephalitis (Blacklaws 2012; Polledo et al. 2011; Torsteinsdottir et al. 2007). Thus, the detection of viral antigen in the perivascular spaces, but not in the neuropil, in the described mild lymphocytic lesions may reflect viral neuroinvasion through the infection of perivascular macrophages or through the entry of infected monocytes into the CNS. Once the infected cells are located in the CNS, viral replication could be controlled by an effective lymphocytic cellular response, but could also progress to severe encephalitis due to dysregulation of the immune response. In the case of lesion progression, immune activation would induce the recruitment and differentiation of more monocytes to macrophages which would enable continuous viral replication and production of cytokines, with an additional non-effective humoral immune response resulting in a severe histiocytic infiltration and lesion of the CNS. This study showed a spectrum of lesions and immunopathological response closely related to the cells and antigen observed in perivascular spaces, and also a possible relation between the spreading of the virus in the neuroparenchyma and the type and severity of the lesion.

However, the material examined in this study was taken from natural cases of ovine VM with varying degrees of severity which had already shown nervous clinical signs, so further understanding of the inflammatory process in VM encephalitis will require analysis of the inflammatory cells as well as expression of cytokines and endothelial adhesion molecules at well-established stages of the disease, including animals in the subclinical phase.

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Simple control strategy to reduce the level of Maedi-Visna infection in sheep flocks with high prevalence values (>90%)

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ABSTRACT

The presence of Maedi-Visna disease in sheep flocks have a significant economic impact, thus the aim of this study was to carry out a serological evaluation of a simple epidemiological control method for ovine Maedi-Visna infection in an intensive dairy flock with high initial seroprevalence. The study population was made up of an initial flock of 197 Spanish Assaf sheep kept in permanent housing using an intensive milking system, and the subsequent five generations of offspring. The control strategy used in this study was mainly based on separating the offspring from the dams after natural intake of colostrum using artificial rearing with milk replacer. Then, replacement groups were formed by these lambs, which were housed in the same shed as the rest of the flock, but in addition, other control measure applied in this study was the isolation of the replacement group from the adults by a barrier (1.20 m high metal sheet) that prevented physical contact between them in the housing, although airspace was shared. Then, replacement groups did not join the adult flock until the onset of first lactation (around 12 months-old). Control measures were first established in 2006 with the second generation offspring, leaving the first replacement group as the control group. Serum samples from all sheep in the flock were screened every six months for antibodies against Maedi-Visna virus using an immunoenzymatic assay. After the application of this control programme, seroprevalence had declined significantly from 93.1% to 54.2% in four years. Results from this study indicate that these control measures may offer a simple alternative for reducing high levels of prevalence in these dairy sheep flocks. Thus, this control strategy is proposed as the first control measures to be taken in flocks with a high prevalence of infection where no other measures could be reasonably applied, followed and expanded by other effective methods when the seroprevalence status of the flock permitting.

1. Introduction

Maedi-Visna virus (MVV), which belongs to the small-ruminant lentivirus family (SRLV), has a worldwide distribution and is responsible for significant production losses in sheep (Peterhans et al., 2004). Maedi-Visna (MV) disease is characterised by a progressive infection in adult sheep resulting in chronic inflammation of the lungs, mamary gland, joints and central nervous system (Dawson, 1987; Lujan et al., 1991).

A MV prevalence study in the north east of Spain showed that 40.7% of randomly sampled animals were seropositive, while 97% of the flocks showed seropositive sheeps (Luján et al., 1993). More recently, the prevalence of MV in the north of Spain has been estimated at 77% in intensive flocks, but specially in half the Spanish Assaf intensive

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flocks this value exceeded 80% (Leginagoikoa et al., 2006b). Several intensive Spanish Assaf flocks present high seroprevalence in Castilla y León (Northwestern of Spain, where this study took place), estimated at 96.8% in an epidemiological study, particularly in those flocks of dairy sheep which are kept in an intensive indoor farming system (Sotelo, 1998).

Natural lactation (colostrum and milk) and direct contact (oral/respiratory transmission) among animals seem to be the two main sources of infection (Berriatua et al., 2003; Blacklaws et al., 2004; Álvarez et al., 2005; Leginagoikoa et al., 2010), and although other routes of Maedi-Visna virus (MVV) transmission, such as vertical and sexual routes, have been reported, they are considered to have limited epidemiological importance (Blacklaws et al., 2004; Broughton-Neiswanger et al., 2010; Romero et al., 2010; Cortez-Romero et al., 2011). Direct contact exposure to infected sheep, and particularly contact between ewe and lamb, seems to be the most important source of infection and an efficient means of horizontal MVV transmission (Houwers et al., 1989; Berriatua et al., 2003; Álvarez et al., 2005; Leginagoikoa et al., 2010), while lactogenic transmission has long been discussed and proposed as a source of MV transmission although making a relatively low contribution to the spread of the disease under natural conditions (Álvarez et al., 2005; Broughton-Neiswanger et al., 2010). Consequently, housing practices, which determine population density have been strongly associated with the spread of MVV (Leginagoikoa et al., 2006a; Pérez et al., 2010).

No commercial vaccines are available to control infection, and to date, monitoring programmes have proved to be the only effective tool in controlling infection. During the Icelandic outbreak, the implementation of a compulsory disease monitoring programme associated with slaughter of affected flocks and replacement with MVV-free animals resulted in effective eradication of MVV (Houwers et al., 1984; Petursson, 1994). Another control strategy was also used successfully in Iceland, based on dividing each affected flock into two groups, one consisting of the adult sheep and the other comprising of a replacement flock formed by offspring which had been removed immediately after birth (Petursson, 1994). Likewise, other less drastic control programmes have been implemented in many countries (the Netherlands, France, Italy, Germany, for instance), mainly based on removing serologically positive animals, as well as separating lambs from seropositive dams immediately after birth for rearing on serologically negative or bovine colostrum (Houwers et al., 1984, 1989).

Sheep culling strategies are feasible in flocks with moderate prevalence (less than 50%), where the number of sheep that become seropositive may be lower than the number of sheep that are annually culled and replaced (Berriatua et al., 2003). However, there are obvious difficulties in applying such strategies, and/or they may be economically unfeasible, in flocks with high seroprevalence as occurs in our region, which reach values of over 80%. Hence, our aim was to evaluate a simple and economical control strategy involving minimal changes in flock management that could easily be followed by farmers.

2. Materials and methods

2.1. Study population, management and control strategy of the study flock

The initial study population in 2005 was a dairy flock consisting initially of 197 adult Spanish Assaf ewes from the Farm at the Veterinary School of the University of León (Spain). Sheep were kept in permanent housing, in a tall, well-ventilated housing, with external access to pasture, having a minimum space of 2.5 m^2 per animal. Rams from the flock were separated from the ewes except in the breeding season. Males varied depending on the year from 3 to 5, and all of them were adults, which came from other farms and changed throughout the study. The handling of this flock during the study was similar to other dairy intensive flocks from the same region.

This initial adult flock and the following five generations of offspring were studied between 2005 and 2010. Replacement sheep came from the female lambs that were born on the farm. The number of lambs that were kept as replacement animals were 100 (replacement group No. 1), 68 (No. 2), 47 (No. 3), 50 (No. 4) and 42 (No. 5), in 2005, 2006, 2007, 2008 and 2009 respectively, not taking into account the immunity status of the dams (seropositive or seronegative to the disease). The rest of the female lambs, which were not included in the replacement groups, and all the male lambs were sold in their first 30 days of age to other farms and were not included in this study. Due to early deaths, the number of sheep that composed each replacement group decreased in the first 18 months of life. The sheep that formed the group of the adult flock each following year were: the adult sheep that have not been sold or have not naturally died from the previous year, together with the replacement group of animals from the previous year (around 12 months old) after their first parturition and beginning of the first lactation.

The handling of the flock was based on a regular management of a dairy intensive flock. The first replacement group from No. 1 was included in this study as a control group and the animals were raised naturally with their dams until 3 months of age. The following groups (No. 2, No. 3, No. 4, No. 5 sheep) were control using the following described control measures: the first one was based on separation of the lambs from the dams after natural intake of colostrum (12–24 hours after birth) and then these lambs were artificially reared with milk replacer. These replacement groups of animals were housed in the same housing as the rest of the flock. But, as a second control measure, these lambs were isolated from the mothers and the adult flock by a barrier (1.20 m high metal sheet) with physical contact between them being prevented, although airspace was shared. These replacement animals did not join the adult flock until the onset of the first lactation. In this study, sheep were considered to have reached adulthood at around 12 months of age, after their first parturition. At this time, they joined the adult group, and were kept under the same management, and were also considered part of the adult group in the data of this study.

2.2. Gross and histopathological studies

Throughout this study, necropsies were carried out on 78 randomly selected adult sheep submitted to the Pathology Diagnosis Unit at the Veterinary College of León. These animals had naturally died on the farm or they were culled due to evident deterioration of their state of health status and humanly euthanised. Thirty-four sheep came from the initial adult flock, 16 sheep came from No. 1 replacement group, 13 from No. 2, 7 from No. 3, 4 from No. 4, and 4 from No. 5. Tissue samples from different organs were taken for microscopic examination, with special attention being paid to the lungs, mammary glands, joints and central nervous system. Sample tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with haematoxylin–eosin and a histopathological study was carried out. Pathologists were blinded to the serological status of the animals and to the PCR results.

2.3. PCR procedures

Genomic DNA from 9 sheep with characteristic MV lung lesions, and from 4 seronegative sheep with no histologically MV lesions was extracted from lung tissue samples using QIAamp® DNA Mini Kit (QIAGEN). Amplification of LTR regions from these samples was performed as described elsewhere (Benavides et al., 2006a,b).

2.4. Serological testing

Serum samples were obtained every six months from all the animals from six months of age for antibody detection. The presence of antibodies against MVV was evaluated using a commercial enzyme-linked immunosorbent assay (ELISA) (Elitest®, Hyphen BioMed, Neuville/Oise, France). The test was carried out following the manufacturer's instructions and the results were reported as positive or negative based on the cut-off value calculated as per the manufacturer's instructions (absorbance 450 nm). The sensitivity and specificity of this ELISA assay have been estimated as 99.4% and 99.3%, respectively (Saman et al., 1999; De Andrés et al., 2005). Specifically, the expected Predictive Value Positive and Predictive Value Negative of this ELISA testing in this flock with the initial prevalence of 89% were 99.9% and 95.3% respectively.

2.5. Statistical analysis

Differences in values were assessed for significance using the chi-squared analysis. The significance level was set at $P > 0.05$.

3. Results

The initial number of 197 adult sheep in 2005 which made up the adult flock varied throughout the study to 175 adult sheep in 2006, 214 adult sheep in 2007, 233 adult sheep in 2008, 204 adult sheep in 2009 and finally 190 adult sheep in 2010. The proportion of the flock replaced each year was 53.1% in 2006, 31.7% in 2007, 15.5% in 2008, 23.0% in 2009, and 22.1% in 2010.

Forty-one of the 78 (52.6%) necropsied animals showed lesions characteristic of MV disease, 38 sheep presenting lung and/or mammary lesions, and 3 sheep with a fatal nervous form and mild lung lesions. Thirty-nine of the 41 animals affected by MV disease yielded positive results in the serological test while two sheep with mild respiratory lesions caused by MV showed negative serological results. Tissue samples of the lung lesions from the 9 animals affected by MV tested by PCR methods yielded positive results, while the PCR results of the 4 seronegative sheep with no MV lesions were negative.

3.1. Serological results

The evolution of the prevalence of MV in the adult flock varied from the beginning of the study in 2005, which was 89.0%. In 2006, MV prevalence in the adult group reached a maximum value of 93.1%, and decreased over the four years after the onset of the controlled programme to 54.2% at the end of the study in 2010. These differences in prevalence values of the adult flock from 2006 to 2010 were found to be statistically significant ($p = 69.85$). Fig. 1 shows the evolution of the prevalence of MV in the adult flock and the respective offspring each year of the study (Fig. 1).

The first replacement group, No. 1, used as control with no changes in management and remaining in close contact with the ewes during natural feeding for their first three months of life, showed an early progressive seroconversion with a prevalence of 47.3% immediately before reincorporation into the adult flock (at 12 months of age), whereas the subsequent replacement groups that had been under control programme (No. 2, No. 3, No, 4 and No. 5) showed statistically significant differences in prevalence rates of 4.4% ($p = 34.97$), 0.0% ($p = 26.24$), 4.2% ($p = 28.52$), 2.4% ($p = 26.28$), respectively (Fig. 1). Table 1 gives prevalence and number of animals comprising each replacement group and their evolution over the first 18 months of life. In addition, there is an early progressive seroconversion of the No. 1 replacement group from six months of age, whereas the beginning of the marked seroconversion of

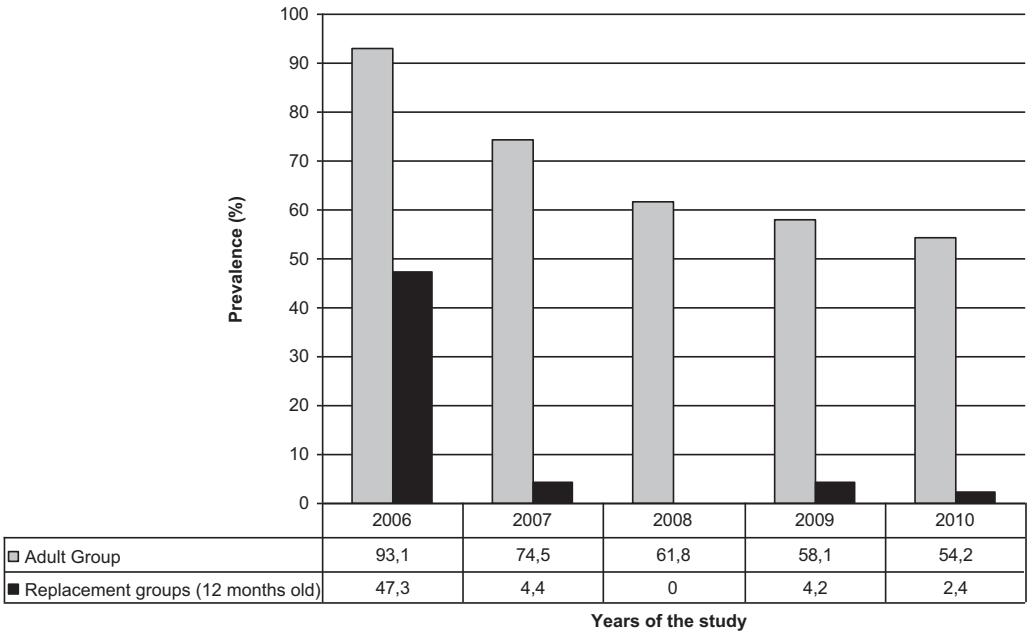

Fig. 1. Evolution of the prevalence of Maedi-Visna infection throughout the years of the study in the adult flock and the consecutive offsprings. Prevalence (*y*-axis) expressed in percentages. Grey columns indicate the percentage of the prevalence in the adult flock each in the corresponding year throughout the four years of the study. Black columns indicate the prevalence of each replacement group at 12 months old, each in the corresponding year during the four years of the study.

Table 1
Prevalence of each replacement group at 6, 12 and 18 months of age.

Replacement group	6 months age		12 months age		18 months age	
	Seropositive sheep[a]	Prev. (%)	Seropositive sheep[a]	Prev. (%)	Seropositive sheep[a]	Prev. (%)
No.1 (2005)	12/100	12	44/93	47.3	47/91	51.4
No.2 (2006)	3/68	4.4	3/68	4.4	5/64	7.8
No.3 (2007)	0/47	0	0/41	0	4/39	10.2
No.4 (2008)	2/50	4	2/47	4.2	4/44	9.1
No.5 (2009)	0/42	0	1/42	2.4	Not sampled	Not sampled

[a] Number of seropositive sheep from the total number of sheep from each replacement group.

the No. 2, No. 3, No. 4 replacement groups was postponed until 18 months of age, after they had joined the adult flock. Replacement group No. 5 showed a prevalence of 2.5% at 12 months of age (last serological test performed in this group of this study). Specifically, Fig. 2 shows how the first replacement group reached 40% prevalence in the first 12 months of life, whereas subsequent groups were around 30 months of age before MV prevalence came close to this value.

Serological results of rams were: 3 seropositive out of 5 in 2005, 4 seropositive out of five in 2006, 4 seropositive out of 5 in 2007, 4 seropositive out of 4 in 2008, 3 seropositive out of 4 in 2009 and 2 seropositive out of 3 in 2010.

4. Discussion

Due to the economic impact of MV on sheep health and production and the ease with which the virus can spread between and within flocks, control programmes may be the best method for effectively and definitively controlling and even eliminating the virus from an area. Epidemiological knowledge, together with the appropriate diagnostic tools and biosecurity practices should be applied, always taking into account the specific conditions and status of each flock.

Testing and culling are the most commonly used strategies to eradicate infection in other countries, and these have been shown to be successful in flocks with moderate prevalence of MV disease (<50%) (Houwers et al., 1984, 1987; Cutlip and Lehmkuhl, 1986; Synge and Ritchie, 2010). However, it not economically feasible to apply these control measures to the intensive dairy flocks with high seroprevalence rates (over 80%) detected in our region. Thus, specific control strategies should be established in these flocks in order to reduce these high levels of prevalence to moderate values of around 40–50%, at which point other, stricter control programmes could feasibly be applied.

Recent studies have shown that seroprevalence increased with flock size and the number of days that sheep are housed (Leginagoikoa et al., 2006a; Pérez et al., 2010; Lago et al., 2012), although VMV may not be efficiently airborne over short distances (Leginagoikoa et al., 2010). For these reasons, a physical separation between adult sheep and offspring sharing the same housing airspace by means of a simple metal sheet is proposed as an affordable and inexpensive control measure, which does not require any other physical space or housing to keep the replacement animals.

Previous studies have shown that ovine colostrum from seropositive ewes can be a major source of MVV infection but that its overall contribution to seroprevalence under natural conditions is relatively low (Álvarez et al., 2005). Moreover, seroconversion has been demostrated to be strongly associated with being born to a seropositive dam, but the risk of seroconversion was similar for lambs fed colostrum and milk from a seropositive or a seronegative dam, independently of the mode of rearing preweaning (Leginagoikoa et al., 2006b). Likewise, seroconversion has been positively associated with increased contact with infected sheep and with the lifetime MV-serological status of the dam. Consequently, when conditions allow efficient horizontal transmission, the evidence that lactogenic infection increases the risk of MV infection seems to be less important (Berriatua et al., 2003). Taking these previous

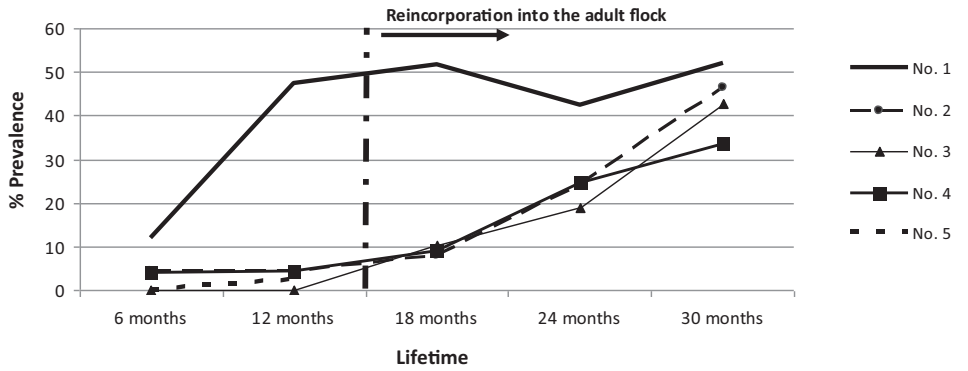

Fig. 2. Evolution of the prevalence in the replacement groups. This figure shows the evolution of the prevalence of infection in percentage (*y*-axis) throughout the study in the each replacement groups from 6 months to 12 months (No. 5), to 24 months (No. 4) and to 30 months of age (No. 1, No. 2 and No. 3).

studies into account and in an attempt to modify flock management as little as possible, another affordable measure proposed in this paper is to avoid initial direct contact exposure (oral/respiratory transmission) between the ewe and the lambs (Houwers et al., 1989; Berriatua et al., 2003; Álvarez et al., 2005; Leginagoikoa et al., 2006b) through early separation of the lambs from the ewes within the first 24 h of life, after natural consumption of colostrum (for easy handling).

Likewise, vertical and sexual transmission, or indirect contact (insemination, embryo transfer, and other iatrogenic causes) seem to play a less important role in the transmission of the infection (Blacklaws et al., 2004), and for this reason no measures were taken in this study to avoid these routes of infection, in order to facilitate flock management. However, these routes of transmission should be considered in order to eradicate the disease.

In this study, the results of PCR and pathological examination indicated the presence of MV infection in the studied flock and its capacity to cause MV disease. The seroprevalence in the entire adult flock decreased from high values of 89.0% in 2005 and 93.1% in 2006, when no possibility existed of implementing standard control programmes, to moderate values of 54.2% in 2010 after applying the control programme proposed in this study. Since there was no marked change in the number of adult sheep, which ranged from 197 (2005) to 190 (2010), this finding, together with the marked decrease in prevalence rates in the replacement groups over the five years, demonstrates that the decrease of the prevalence rate in the flock was not due to the animal replacement rate.

There were also clear differences between the prevalence rates in the No. 1 replacement group (47.3%) at one year of age before being reincorporated into the adult flock, and those in the subsequent replacement groups (No. 2, No. 3, No. 4 and No. 5), which did not reach 5%. These data would mean that these first three months of the lambs' lifetime are critical in the transmission of the disease, probably due to infection through ingestion of infected colostrum/milk and to the horizontal transmission by the close contact with the mothers. Thus, the avoiding of these ways of transmission as is put forward in this study results in a minimal early infection of the offspring. These low levels of seroconversion in groups No. 2, No. 3, No. 4 and No. 5 could be associated with the ingestion of infected colostrum or vertical transmission (Álvarez et al., 2005, 2006). Thus, the used in breeding of seropositive rams could have contributed to early infections of newborn lambs in this study. Vertical transmission was not considered in the control measures applied in this study in order to simplify handling, but should be taken into account later on in order to eradicate disease from the flock.

In this study it could be detected that the main seroconversion in the control replacement groups No. 2, No. 3, No. 4 occurred after they joined the adult flock and continue along the study, so the infection between adult sheep occurred in an important way. Moreover, the data regarding the delay, from 12 months to 30 months of age, in seroconversion to 40% prevalence in the controlled groups compared with the non-controlled group is very significant, especially in intensive dairy sheep flocks. Since the longevity of these highly productive sheep is on average 4 yrs of age, and bearing in mind the chronic development of MV disease after infection, manifestation of the disease with the consequent decrease in production as well as premature death would be minimised.

Subsequently, following the decline in prevalence values (54.2%), other stricter control measures could be established, in order to obtain lower prevalence values. Thus, from this point in which the flock has reached a moderate level of prevalence, it should be possible to reduce flock seroprevalence progressively by selectively culling seropositive sheep and replacing them with offspring from seronegative ewes, as has been previously proposed (Houwers et al., 1984, 1987; Cutlip and Lehmkuhl, 1986; Berriatua et al., 2003; Synge and Ritchie, 2010). Likewise, isolating these seronegative newborn lambs from the rest of the flock would be very useful (Cutlip and Lehmkuhl, 1986; Reina et al., 2009), and avoiding the ingestion of colostrum by replacing it with bovine colostrum or colostrum from seronegative ewes (Peterhans et al., 2004). The serological status of the rams should also be taken into account in further control strategies in order to only use seronegative ones in breeding. Then, a measure, which could be incorporated into the final control programme in order to enable early detection of the infected animals and rapid erradication of the infection from the flock could be a combination of serology and polymerase chain reaction (PCR) (Brinkhof et al., 2010). Future objectives include the possibility of officially certifying the MV-free status of flocks as part of a strategy to obtain healthy flocks with respect to MV, enabling farmers to avoid introduction or reintroduction of the infection and the consequent economic loss entailed.

Since successful results were obtained, these simple control measures are proposed as an initial simple control programme on farms with a high level of MV infection where no other measures could reasonably be applied.

5. Conclusion

The aim of this study is to design a MV control programme for implementation in flocks with high prevalence values where the application of other previously described control measures is completely impractical. The results demonstrate that the control programme designed can be applied successfully to reduce high MV prevalence (about 90%) in a dairy sheep flock to more moderate values, and has the potential for extension to larger scale operations. Following this decline in prevalence values, other stricter control measures should be established in order to obtain low prevalence values or even eradicate the infection from the flock.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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