

Short Communication

Successful induction of ovine pulmonary adenocarcinoma in lambs of different ages and detection of viraemia during the preclinical period

Daniela Salvatori,¹ Lorenzo González,² Patricia Dewar,¹ Christina Cousens,¹ Marcelo de las Heras,³ Robert G. Dalziel⁴ and J. Mike Sharp²

Correspondence

Daniela Salvatori
salvd@mri.sari.ac.uk

^{1,2}Moredun Research Institute¹ and Veterinary Laboratories Agency (VLA-Lasswade)², Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, UK

³Universidad de Zaragoza, Facultad de Veterinaria, 50013 Zaragoza, Spain

⁴University of Edinburgh, Division of Veterinary Biomedical Sciences, Edinburgh EH9 1QH, UK

Ovine pulmonary adenocarcinoma (OPA) can be reproduced consistently in neonatal lambs by intratracheal injection of inocula containing jaagsiekte sheep retrovirus (JSRV). In this study, clinical disease, confirmed pathologically as OPA, was induced in a high proportion of lambs that had been inoculated intratracheally with infectious lung fluid at 1, 3 and 6 months of age. The incubation periods, however, were longer in these three age groups than in 1-week-old lambs that were used as controls. Viraemia was detected in all age groups before onset of clinical signs, but occurred later in older animals. These results suggest an age-dependent susceptibility to OPA that could be determined by the availability of JSRV target cells in the ovine lung. The feasibility of inducing OPA in older lambs and detecting JSRV viraemia in preclinical stages enables improved studies on the pathogenesis, assessment of vaccines, diagnosis and control of the disease.

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Ovine pulmonary adenocarcinoma (OPA) is a contagious lung cancer that is caused by an exogenous betaretrovirus, jaagsiekte sheep retrovirus (JSRV), which is distinct from the transcriptionally active endogenous retroviral sequences that are present in the ovine genome (York *et al.*, 1992; Palmarini *et al.*, 2004). The mechanism by which the virus causes oncogenic transformation of lung epithelial alveolar type II (ATII) and Clara cells is still not understood completely. Whilst natural OPA has a long incubation period, with most clinical cases occurring in 2–4-year-old animals (Sharp & DeMartini, 2003), the disease can be induced experimentally very rapidly and efficiently by intratracheal inoculation of newborn lambs with concentrated lung fluid (LF) (Verwoerd *et al.*, 1980; Sharp *et al.*, 1983; De las Heras *et al.*, 2003). However, following a similar intratracheal challenge of 10-week-old lambs with tumour extract, only 25% of them developed scattered neoplastic nodules and none showed clinical disease after 8 months (DeMartini *et al.*, 1987; Rosadio *et al.*, 1988). Similarly, in early transmission studies that were performed in sheep older than 1 year by parenteral inoculation or aerosol exposure, clinical disease was seldom achieved, with incubation periods usually longer than 1 year and pathological changes consisting of confined small tumour nodules (Dungal, 1946; Wandera, 1968).

The neonatal lamb model has been very useful in determining the aetiological role of JSRV in OPA and some aspects of its pathogenesis, but is unsuitable for evaluating the efficacy of potential vaccine preparations and studying pathogenic mechanisms of JSRV infections that occur later in life. These studies also have been hampered by the absence of detectable antibodies to JSRV in affected animals (Sharp & Herring, 1983; Ortín *et al.*, 1998).

The aims of the present study were to investigate whether JSRV infection, OPA lesions and clinical disease could be induced in older lambs and to determine the dynamics of the infection by PCR examination of peripheral blood samples.

Four groups of Scottish Blackface lambs were inoculated with JSRV-containing LF, as confirmed by Western blot and RT-PCR (Palmarini *et al.*, 1995). Ages at inoculation were: 1 week ($n=5$), 1 month ($n=10$), 3 months ($n=10$) and 6 months ($n=10$). All animals were from an OPA-free flock at Moredun Research Institute, UK. Six lambs from the same source were used as unchallenged controls.

LF collected from sheep that were naturally affected with OPA was clarified and stored at -70°C . In order to remove variation in JSRV titres between batches of LF and to

ensure that each lamb received the same inoculum, several batches of LF from different sheep were thawed, pooled together, aliquotted and stored at -70°C . Immediately before inoculation of the lambs, the appropriate number of aliquots were thawed and concentrated to give a 12.5-fold concentrate of the original volume (Sharp *et al.*, 1983). Each lamb in each age group received 5 ml of this concentrated LF pool by the intratracheal route, as described previously (Sharp *et al.*, 1983).

Peripheral blood samples were collected into EDTA tubes immediately before challenge and at intervals until necropsy. Peripheral blood leukocytes (PBLs) were obtained by centrifugation after lysis of erythrocytes (García-Goti, 1999) and the cell pellet was stored at -70°C . DNA extraction was performed by using a DNeasy Tissue kit (Qiagen) following the manufacturer's protocol for animal blood. Blood samples from OPA-free Icelandic sheep were processed simultaneously as negative controls.

A single-round PCR to amplify the U3 region of JSRV was developed, based on the previously reported primers PI and PIII (Palmarini *et al.*, 1996). The new PCR was validated by using DNA from the OPA cell line JS7 (Jassim *et al.*, 1987; DeMartini *et al.*, 2001), diluted in a background of 600 ng DNA from Icelandic sheep PBLs. Optimized PCR conditions were: 5 μl $10\times$ PCR buffer (Qiagen), 200 μM dNTPs (Roche Diagnostics), 200 nM each primer (MWG) and 1.25 U HotStarTaq polymerase (Qiagen) were added to ultra-pure water (Sigma) to a final volume of 50 μl . To this pre-mix, 600–800 ng test DNA was added and incubated at 94°C for 16 min, followed by 35 cycles at 94°C for 30 s, 59°C for 30 s and 72°C for 30 s, with a final extension step of 5 min at 72°C . This JSRV-U3-PCR successfully amplified 10–100 copies of JSRV template that were integrated into JS7 genomic DNA (data not shown). The test samples were analysed in triplicate and considered to be positive if one or more of the replicates gave a band of the correct molecular mass. To confirm the integrity of the DNA, samples that were negative in the JSRV-U3-PCR were tested for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Palmarini *et al.*, 1996).

Animals were monitored daily after experimental infection for clinical signs of respiratory disease that might be suggestive of OPA. As soon as these were identified, lambs were killed humanely. Lambs that did not show any clinical signs were kept for a maximum of 7 months post-infection (p.i.) and then culled. Unchallenged control animals were culled at the end of the experiment, at the same time as the last inoculated lambs. At necropsy, examination of the lungs was performed to identify any gross lesions that might be suggestive of OPA. Neoplastic-like areas or, in their absence, a range of tissue sites from all pulmonary lobes were taken for microscopic examination. Samples were fixed in 10% neutral-buffered formalin, processed routinely, sectioned at 5 μm and stained with haematoxylin and eosin (HE). Sections from selected lung samples were subjected to immunohistochemical examination for JSRV

capsid protein (JSRV-CA) and JSRV surface glycoprotein (JSRV-SU) by using a newly developed antiserum, which was prepared by immunizing rabbits with glutathione S-transferase-SU (GST-SU) and His-tagged SU proteins in Freund's incomplete adjuvant. Recombinant fusion proteins were made by cloning and expressing nt 5557–6483 of JSRV (GenBank accession no. M80216) following standard molecular biology procedures (Sambrook *et al.*, 1989). The fragment inserted in pGEX1 λ T (Pharmacia) was expressed in *Escherichia coli* cells to generate GST-SU, which was purified by using glutathione/Sepharose beads (Invitrogen). The fragment inserted in pIVEX2.4a (Roche Molecular Biochemicals) was expressed as a His-tagged protein by using the cell-free translation system RTS-500 (Roche Molecular Biochemicals), purified by using ProBond nickel resin (Invitrogen) and eluted following denaturing conditions according to the manufacturer's protocol. Both antibodies were used at 1/1000 dilutions as described previously (Palmarini *et al.*, 1995). Samples of lung and mediastinal lymph node were taken for JSRV-U3-PCR analysis.

None of the unchallenged control lambs developed clinical signs of respiratory disease and their PBLs were negative by JSRV-U3-PCR throughout the experiment. When culled at 14 months of age, neither histopathological lesions of OPA nor proviral DNA was detected in lung or mediastinal lymph-node samples.

In four of five 1-week-old lambs, severe clinical signs that were suggestive of OPA were observed at 70–74 days p.i. (Fig. 1), whereas the other lamb (1W1; Fig. 2) died at 28 days p.i. of an unrelated illness. All five lambs showed

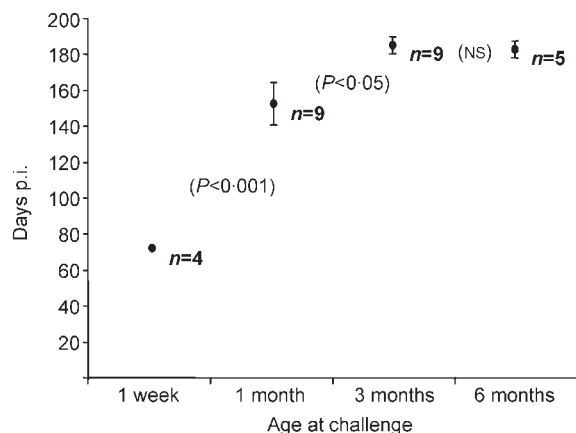


Fig. 1. Incubation period of experimental OPA depending on age at challenge. Incubation period (days from experimental infection to post-mortem) of OPA in four groups of sheep that were challenged at different ages, all of which showed clinical signs of the disease and histopathological confirmation of pulmonary neoplasia. Results are expressed as mean values; bars indicate SEM; statistical differences between groups appear in parentheses. NS, Not significant.

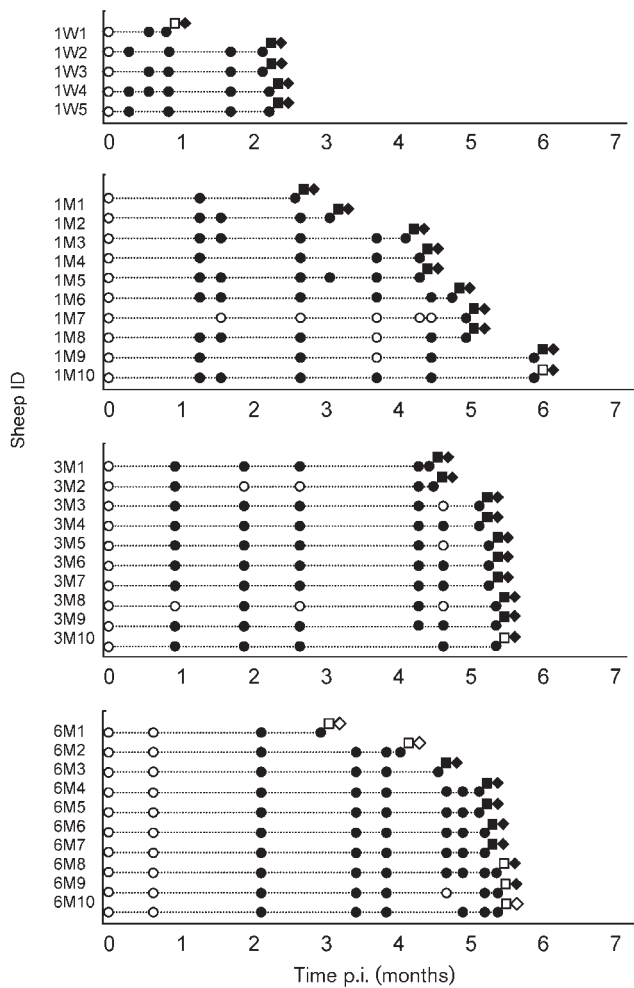


Fig. 2. Evolution of JSRV viraemia as detected by U3 PCR (○, negative; ●, positive) during the course of experimental infection and at culling such showing clinical signs of OPA (□) or not showing such signs (□), and in which gross and/or histopathological lesions of OPA were confirmed (◆) or absent (◇).

gross and microscopic lesions of OPA (Fig. 2) and were consistently positive after infection by JSRV-U3-PCR (Fig. 2).

Nine out of ten 1-month-old lambs showed clinical signs of OPA, with incubation periods ranging from 92 to 209 days (mean, 152 days; Fig. 1). The remaining lamb (1M10; Fig. 2) was asymptomatic when culled at almost 7 months p.i. All ten lambs had macroscopic pulmonary lesions that were confirmed histologically as OPA (Fig. 2) and were JSRV-U3-PCR-positive throughout the incubation period, with the exception of one (1M7; Fig. 2).

Nine out of ten 3-month-old lambs developed signs of OPA, with incubation periods ranging from 159 to 192 days (mean, 185 days; Fig. 1). The other animal (3M10; Fig. 2) was asymptomatic when culled at 192 days p.i. Macroscopic lung lesions were confirmed histologically as OPA in all ten

lambs. Whilst JSRV viraemia was detected consistently in six lambs, including the one that remained asymptomatic, the other four (3M2, 3M3, 3M5 and 3M8; Fig. 2) were JSRV-U3-PCR-negative on occasions during the experiment.

Five out of ten 6-month-old lambs developed clinical OPA with incubation periods ranging from 164 to 169 days (mean, 182 days; Fig. 1) and, amongst the other five sheep, three (6M8, 6M9 and 6M10) were asymptomatic when culled at 190–192 days p.i. All five clinically affected sheep and two asymptomatic lambs showed pathological evidence of neoplasia, although in one of them (6M9; Fig. 2), only microscopic lesions were detected. Two lambs (6M1 and 6M2; Fig. 2) died early from OPA-unrelated conditions; in these and in one of the asymptomatic culls (3M10; Fig. 2), neither gross nor microscopic lesions of OPA were found. Within this group of 6-month-old lambs, PBLs from all animals were JSRV-U3-PCR-negative at 3 weeks p.i. but, with the exception of a single sheep (6M9; Fig. 2), they were consistently positive thereafter.

Regardless of age at challenge, all sheep with histologically confirmed OPA showed the same pattern of neoplasia, which consisted predominantly of papillary adenocarcinoma of the alveoli (Fig. 3a, b). Immunohistochemical examinations showed JSRV-CA protein in the cytoplasm of a proportion of ATII neoplastic cells and nodules with the features described in previous studies (Palmarini *et al.*, 1995; Platt *et al.*, 2002). Detection of immunohistochemical reactivity to JSRV-SU was consistent in epithelial cells lining neoplastic alveoli (Fig. 3c) and in polypoid structures in the bronchioles (Fig. 3d). Immunolabelling was strongest at the apical surface of the cells.

JSRV was detected by U3-PCR in all mediastinal lymph-node and lung-tissue samples that were collected from the 35 JSRV-challenged sheep.

The present study demonstrates clearly that JSRV infection can be induced in 100 % of lambs aged 1–6 months at the time of inoculation and that a high proportion of these animals develop clinical signs (62–90 %) and lesions (87–100 %) of OPA. This is in contrast to a previous study on a much smaller scale, in which only a proportion of 10-week-old lambs developed neoplastic lesions (Rosadio *et al.*, 1988). One explanation for this discrepancy and for the success of the current experiment would be a large amount of JSRV in the inoculum, as LF contains more JSRV than tumour extracts (Herring *et al.*, 1983) and JSRV dose and incubation period are correlated inversely (Verwoerd *et al.*, 1980). Nevertheless, some findings in the present study support the notion of an age-related resistance to OPA. Firstly, within those animals that developed clinical OPA, the incubation period was significantly shorter ($P < 0.001$) for lambs that were inoculated at 1 week of age than for older lambs (Fig. 1) and it was also shorter ($P = 0.03$) for animals that were challenged at 1 month than for those inoculated at 3 months. Secondly, the development of lesions was delayed in older lambs, as indicated by three

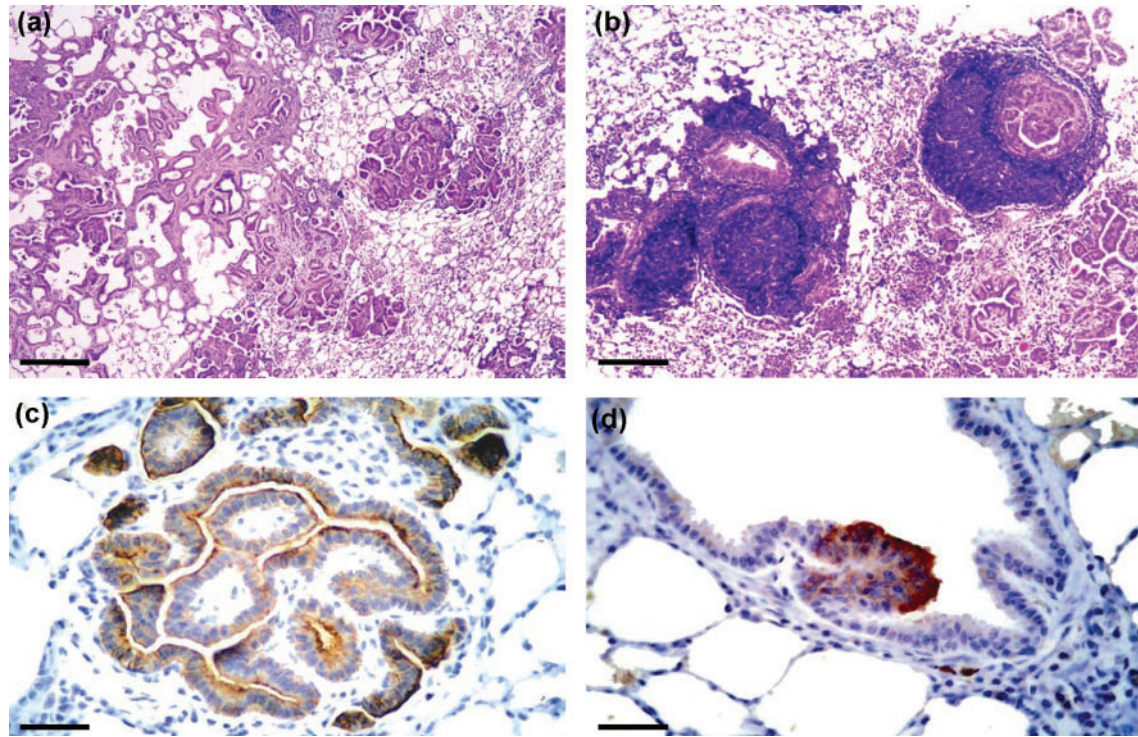


Fig. 3. Histopathology and immunohistochemistry of experimental OPA. (a) Co-existence of papillary and acinary forms of the tumour and presence of desquamated alveolar macrophages in the lung of a lamb inoculated at 1 month of age (HE staining). Bar, 400 μm . (b) Papillary neoplastic proliferations involve alveoli and bronchioles, which also show hyperplasia of associated lymphoid tissue. Para-adenomatous change is conspicuous. Lamb was inoculated at 1 month of age (HE staining). Bar, 200 μm . (c) Papillary alveolar tumour in a lamb challenged at 3 months of age, showing strong immunoreactivity for JSRV-SU protein mainly in the apical border of transformed ATI cells. Haematoxylin counterstaining. Bar, 60 μm . (d) Proliferating cells in a bronchiole show immunoreactivity for JSRV-SU protein (most evident in the apical cytoplasm), whereas non-transformed cells are negative. Note some immunolabelled cells in the interstitium. Lamb was inoculated at 6 months of age. Haematoxylin counterstaining. Bar, 60 μm .

asymptomatic lambs in the oldest age group that had no evidence of OPA lesions and a fourth lamb with only microscopic lesions (Fig. 2). Finally, detectable viraemia occurred later in the group of 6-month-old lambs than in the group of 1-week-old lambs (Fig. 2), and possibly also later than in the 1- and 3-month-old animals, although straight comparisons cannot be made with these two groups, as their first PCR testing on PBLs was slightly delayed.

As the same inoculum was used for all 35 lambs, our results suggest that age at infection has an effect on the development of OPA; this is particularly noticeable between 1 week and 1 month of life.

Other examples of age-related susceptibility to retroviral infections include feline leukemia virus (Hoover *et al.*, 1976; Flynn *et al.*, 2000), certain avian leukosis viruses (Pizer *et al.*, 1992; Stedman *et al.*, 2001), visna/maedi virus (Lairmore *et al.*, 1986; Andresson *et al.*, 1993) and Mason-Pfizer monkey virus (Fine *et al.*, 1975). Whilst, for some of these infections, differences in the ability of neonates and older animals to mount immune responses could account for an

age-related susceptibility, this is unlikely for OPA, in view of the lack of a specific humoral immune response to JSRV (Sharp & Herring, 1983; Ortín *et al.*, 1998).

A more plausible explanation for the age effect in OPA would be that the target cells for primary JSRV infection and oncogenesis, ATII pneumocytes and Clara cells of the bronchioles, are present in higher numbers in newborn lambs than in older animals. In rodents and ruminants, the ratio of type I pneumocytes to ATII cells at birth is around 1:2, with ATII pneumocytes accounting for approximately 60% of alveolar cells (Weibel & Taylor, 1996; Kahwa *et al.*, 1997; Otto, 1997). During the first month of life, the number of ATII cells declines progressively and dramatically, so that they represent only 6% of alveolar cells at 7 days, 1% at 3 weeks and 0.2% at 1 month, with no further significant changes occurring later (Wright & Alison, 1984). Similarly, Clara cells retain fetal characteristics in calves and goat kids that are up to 30 days old, but lose them and become fully differentiated after this time (Castleman & Lay, 1990; Kahwa *et al.*, 2000). We hypothesize that following intratracheal inoculation of

JSRV, primary virus replication would occur in the ATII and Clara cells and that the magnitude of such replication would depend on the availability of these target cells. Thus, higher viral production could be expected in newborn lambs, leading to earlier detection of viraemia and development of neoplastic lesions (and hence a shorter incubation period), and vice versa in older lambs.

In conclusion, we have developed a new experimental model to induce JSRV infection and OPA in aged lambs, which can be monitored by an efficient PCR technique in blood samples. Apart from providing new insights into the pathogenesis of the disease by confirming an age effect in the timescale of OPA-related events, the model will be useful to test the efficacy of potential vaccines. Finally, our PCR results reinforce previous studies on the detection of JSRV infection in subclinically infected sheep (García-Goti, 1999; González *et al.*, 2001), providing an opportunity for future epidemiological research.

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