Systemic immune responses following infection with Jaagsiekte sheep retrovirus and in the terminal stages of ovine pulmonary adenocarcinoma

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Jaagsiekte sheep retrovirus (JSRV) is the aetiological agent of ovine pulmonary adenocarcinoma (OPA). To monitor changes in cellular immune function during JSRV infection, lymphoproliferation in response to various mitogens was measured in the blood of conventionally housed and specific-pathogen-free lambs experimentally infected with JSRV until the development of OPA and compared with uninfected control lambs. In addition, blood samples collected from adult field cases in the terminal stages of OPA and control adult sheep were compared. No difference in the proliferative response to phytohaemagglutinin and pokeweed mitogen between the animal groups was detected. In contrast, reduced responses to concanavalin A stimulation were demonstrated in the JSRV-inoculated lambs, prior to the onset of clinical disease, and also in the terminally ill adult sheep. Peripheral blood leukocytes were monitored to identify phenotypic frequency alterations. The CD4 lymphocytopenia and neutrophilia reported previously in adult OPA cases were demonstrated but similar phenotypic changes were not identified during experimental infection.

The β-retrovirus Jaagsiekte sheep retrovirus (JSRV) (Sharp et al., 1983) is the confirmed aetiological agent of ovine pulmonary adenocarcinoma (OPA), known previously as sheep pulmonary adenomatosis and ovine pulmonary carcinoma (Palmarini et al., 1996a, 1999). The immunological response to JSRV infection is poorly understood. No JSRV-specific humoral response has been detected (Sharp & Herring, 1983; Ortin et al., 1998). The cell-mediated response to JSRV infection is, as yet, unknown, although CD4 lymphocytopenia and neutrophilia have been demonstrated in the peripheral blood of adult sheep during the terminal stages of OPA (Rosadio & Sharp, 1992; Holland et al., 1999). A disseminated infection occurs as an early event, with viral RNA and proviral DNA found in cells of the immune system. Although very few cells are infected, the largest provirus load is in the monocytes/macrophages, followed by B cells, then T cells. However, there is no evidence of virus transformation of any of these cell types (Palmarini et al., 1996b; Holland et al., 1999).

The lack of appropriate antigens and JSRV-specific immunological assays has impeded investigations of immune responses during tumourigenesis in affected flocks. However, OPA can be induced experimentally in neonatal lambs by intratracheal inoculation with virus purified from tumour (Martin et al., 1976), lung fluid (Sharp et al., 1983) or infectious JSRV molecular clones (Palmarini et al., 1999; DeMartini et al., 2001). Thus, monitoring phenotypic and functional changes in the peripheral blood during experimentally induced tumourigenesis is possible.

The aim of this study was to investigate the systemic immune responses at all stages of JSRV infection, through tumourigenesis and into the terminal stages of OPA, and to establish if changes such as the CD4 lymphocytopenia and neutrophilia reported in adult OPA cases (Rosadio & Sharp, 1992; Holland et al., 1999) were early events during JSRV infection, a consequence of tumourigenesis, or were demonstrated only in terminally ill animals. Moreover, the investigation was extended to identify alterations in cellular immune function attributable to JSRV infection.

The immune status in the terminal stages of OPA was determined by the lymphoproliferative responses to the mitogens phytohaemagglutinin (PHA), pokeweed mitogen (PWM) and concanavalin A (ConA) and phenotypic characteristics in a single blood sample collected from ten adult field cases (brought from various Scottish farms), compared with those of ten uninfected adult sheep (from stock at the Moredun...
Research Institute). Systemic immune responses during the pre-clinical stages of disease were monitored in serial blood samples collected from six neonatal Dorset lambs inoculated intratracheally with JSRV purified from lung fluid, as described previously (Sharp et al., 1983), and compared with four, age-matched, uninfected control lambs. To exclude the effects of external influences and to eliminate secondary infections, the investigation was extended to include specific-pathogen-free (SPF) lambs (six JSRV-inoculated and four control Suffolk-X lambs).

In all experiments, venous blood samples were collected into sterile, preservative-free, heparinized vacutainers (10 units heparin per ml of blood). Aliquots were removed for neutrophil counts. To adequately remove the large neutrophil population present in the blood of adult OPA cases, it was necessary to centrifuge the whole-blood sample for 15 min at 300 g. To adequately remove the large neutrophil population, the investigation was extended to include specific-pathogen-free (SPF) lambs (six JSRV-inoculated and four control Suffolk-X lambs).

When symptoms of OPA were apparent, the animals were humanely killed by an overdose of pentobarbitone (Rhone Merieux) and necropsy was performed. Diagnosis of OPA, based on the characteristic gross and histopathological lesions described elsewhere (Sharp & Angus, 1990), confirmed the disease status of the experimentally infected lambs and adult sheep. For the conventionally housed lambs, OPA was confirmed in three lambs at 12 weeks, one lamb at 16 weeks and one lamb at 20 weeks of age. In the SPF experiment, OPA was confirmed in one lamb at 9 weeks, one lamb at 18 weeks and one lamb at 20 weeks of age. In each experiment, two control lambs were culled at 16 weeks and the remaining two control lambs were culled at 20 weeks; none of the lambs showed lesions of OPA.

To demonstrate that phenotypic frequency alterations occur in adult OPA cases but not during experimental JSRV infection, PBMC phenotyping by indirect immunofluorescence was performed using standard laboratory procedures (Bach et al., 1995; Lloyd et al., 1995). Cells were immunolabelled with a panel of mouse anti-sheep monoclonal antibodies specific for CD2 T cells (135A), CD4 T cells (17D), CD8 T cells (SBU-T8), $\gamma\delta$ T cells (86D), B cells ($\alpha$-light chain VPM8) and MHC class II antigens (VPM54); all antibodies were supplied by Professor John Hopkins, University of Edinburgh, Edinburgh, UK). The secondary antibody was an FITC-conjugated rabbit anti-mouse immunoglobulin (DAKO) and the results were determined by flow cytometry (EPICS XL-MCL Coulter Electronics). The percentage of neutrophils present in the white blood cell population was determined by flow cytometry based on cell size and granularity, following red blood cell lysis with Erthyrolyse (Serotec). The mean percentage for each animal group was determined $\pm$ SEM. Student's two-tailed $t$-test determined the level of significance between the groups.

In adult sheep with OPA, a significant decrease ($P = 0.02$) in the percentage of positively immunolabelled CD2 T cells ($42 \pm 3\%$) was detected in the blood compared with uninfected controls ($52 \pm 3\%$). The depletion in the T cell population was further characterized by a CD4 lymphocytopaenia, with a significantly reduced ($P = 0.004$) percentage of CD4 T cells ($16 \pm 2\%$) detected in the adult OPA cases compared with the control sheep ($24 \pm 2\%$). No significant frequency alterations were identified for CD8 T cells, $\gamma\delta$ T cells, B cells or MHC class II antigens. The percentage of neutrophils present in the blood showed a significant increase ($P = 0.05$) in the terminally ill adult OPA cases ($52 \pm 6\%$) compared with the control sheep ($33 \pm 6\%$). In contrast, no statistically significant phenotypic frequency alterations were identified, at any time during the 20 weeks after inoculation, in the blood of the experimentally JSRV-infected lambs (conventionally housed and SPF) in comparison with their respective controls.

To show that ConA lymphoproliferative responses are reduced in experimentally JSRV-infected lambs and adult sheep with OPA, the optimum concentration for each mitogen, 12 $\mu g/ml$ for PHA, 15 $\mu g/ml$ for ConA and 2 $\mu g/ml$ for PWM (Sigma), was added to $10^6$ PBMCs per ml RPMI 1640 medium. Control samples (excluding lectin) were also prepared. Lymphoproliferation was performed at $2 \times 10^6$ PBMCs per well (in triplicate) in accordance with standard laboratory protocols (Kilpatrick, 1998) for a total of 96 h, with the addition of $0.7 \mu Ci$ per well [methyl-$^3$H]thymidine (sp. act. 2.0 Ci/mmol, Amersham) for the final 16 h. $[^3H]$Thymidine uptake was measured as c.p.m. For each sample, the mean c.p.m. of the replicates was calculated and the control mean c.p.m. subtracted. The resulting individual values were used to calculate the mean c.p.m. of each animal group $\pm$ SEM. Student's two-tailed $t$-test determined the significance of the differences between the mean c.p.m. of the different groups.

No significant difference in the level of response to PHA stimulation was identified between the adult OPA sheep and the controls. In contrast, a significantly reduced response ($P = 0.03$) to ConA stimulation was evident in the PBMCs of adult OPA cases ($10955 \pm 2532$ c.p.m.), which was only 58% of the response observed in the controls ($19000 \pm 2426$ c.p.m.). No difference in response to PHA (Fig. 1a) or PWM (Fig. 1b) stimulation was detected between JSRV-inoculated and conventionally housed control lambs. However, the infected lambs were significantly ($P = 0.002–0.008$) less responsive to ConA stimulation than the control animals (Fig. 1c). This observation was detected first when the lambs were 8 weeks old; at that time, the response in the JSRV-infected group was only 33% of that in the control lambs. Reduced responses to ConA stimulation in excess of 75% were demonstrated in the infected lambs until the end of the experiment at 20 weeks. The lymphoproliferative responses of the infected SPF lambs also
demonstrated a reduced response to ConA stimulation (results not shown).

JSRV CA-specific antibodies were not detected by indirect sandwich ELISA. Plasma collected from all the sheep were tested for the presence of JSRV CA antibodies by ELISA. A recombinant JSRV CA-coating antigen was prepared by cloning part of the JSRV gag gene (nucleotide sequence published by York et al., 1991) into the plasmid pBAD/His A (Invitrogen) and expressed in Escherichia coli LMG 194 cells. The plasmid without the CA insert was used as a negative control-coating antigen. Sheep anti-CA serum, as a positive control, was generated according to Palmarini et al. (1995) with some minor adaptations. Specific absorption values were calculated by subtracting the control values from the test values. The cut-off point to define seropositivity was set as the mean $A_{450}$ of a negative control serum (pre-immunization serum) plus three times the standard deviation of the mean (four replicates); none of the plasma samples from the experimental lambs or adult sheep with OPA contained any CA antibodies.

The present investigation of lymphoproliferative responses to mitogen stimulation has revealed for the first time an alteration in host cellular response due to the presence of JSRV. A reduced response to ConA stimulation was evident in the PBMCs of experimentally JSRV-infected lambs as an early event during tumourigenesis, being apparent from 8 weeks of age, prior to clinical disease. Moreover, this functional alteration was evident in the adult OPA sheep tested.

There are many examples of retrovirus-encoded proteins with immunosuppressive potential but, during JSRV infection, there has been no evidence of virus transformation of immune cells, although a disseminated infection is evident (Holland et al., 1999). Despite the low virus load, the results of this study show that the presence of JSRV does alter cellular function. During the early stages of human immunodeficiency virus (HIV) infection, when only a small percentage of CD4 lymphocytes are infected, the cellular function of the T helper cell population is adversely affected prior to their depletion (Miedema et al., 1988). It has been established that retrovirus suppression can be selective for one mitogen without the response to other mitogens being affected. For example, HIV-1 inhibits PHA-induced lymphoproliferation but does not affect the response to ConA stimulation (Mann et al., 1987). The transmembrane protein p15E from murine leukaemia virus (Ruegg et al., 1989; Schmidt et al., 1987) and feline leukaemia virus (Mathes et al., 1978, 1979) induces immunosuppression by preventing aggregation of ConA receptors, suggesting that expression of this protein interferes with the redistribution of the cytoskeleton (Dunlap et al., 1979). Therefore, although immune cells may not be transformed by JSRV, specific changes in glycoprotein-bound carbohydrate residues during JSRV infection may be involved.

If no JSRV-specific adaptation at the cell surface occurs, then virus modification of post-recognition events, such as transport mechanisms and the intracellular pathways utilized during mitogenesis, may be implicated. Recent studies have identified that the cytoplasmic moiety of the JSRV envelope is sufficient to transform rat and mouse NIH 3T3 cells (Maeda et al., 2001; Rai et al., 2001). Phosphorylation of protein kinase B was detected in all JSRV-transformed cells, which would indicate that expression of the JSRV envelope activates the phosphatidylinositol 3-kinase (PI-3K) pathway (Palmarini et al., 2001). PI-3K phosphorylation of protein kinase B is known to inhibit apoptotic mechanisms and is essential for the transition from G1-to-S phase during the lymphoproliferative response to several mitogens (Roche et al., 1994). Therefore, signal transduction and regulatory mechanisms may be influential by contributing not only to the neoplastic process but also in determining the altered cellular response of JSRV-infected sheep.

Our knowledge of the PBMC phenotypic characteristics already identified in OPA cases was extended during this
present study. No evidence of the CD4 lymphocytopenia and neutrophilia, detected in the blood of adult sheep during the terminal stages of OPA (Rosadio & Sharp, 1992; Holland et al., 1999) and confirmed in this study, was revealed during experimental disease. This may suggest that these changes do not occur in direct response to JSRV infection but are a consequence of increased susceptibility to secondary infections encountered as the health of the animal declines.

In adult OPA cases, no evidence of circulating JSRV-specific antibodies has been recorded either by Western blotting or by ELISA (Sharp & Herring, 1983; Ortin et al., 1998). In addition, this study demonstrated a lack of specific antibodies for the JSRV CA during acute experimental JSRV infection and OPA development.

With no circulating JSRV-specific antibodies detected and phenotypic alterations only demonstrated in adult OPA cases, the reduced response to ConA stimulation indicates an alteration in systemic immunity. This change is an early event during JSRV infection, sustained through tumourigenesis and into the terminal stages of OPA.

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References


Ruegg, C. L., Monell, C. R. & Strand, M. (1989). Identification, using synthetic peptides, of the minimum amino acid sequence from the...


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