THE POSSIBLE INVOLVEMENT OF IMMUNOSUPPRESSION CAUSED BY A LENTIVIRUS IN THE AETIOLOGY OF JAAGSIEKTE AND PASTEURIELLOSIS IN SHEEP

M. S. MYER(1), H. F. A. K. HUCHZERMeyer(2), D. F. YORK(2), P. HUNTER(2), D. W. VERWOERD(2) and HELEN M. GARNETT(3)

ABSTRACT


A South African isolate of ovine lentivirus was shown to cause a mild immunosuppression in sheep, reflected by a reduced delayed hypersensitivity reaction. This effect, measured in terms of skin swelling after intradermal inoculation with tuberculin, showed a positive linear relationship with the latency period before the appearance of jaagsiekte symptoms in animals co-infected with JSRV, as well as with the activity of monococytes. In a parallel study, increased susceptibility of lentivirus-infected sheep to infection with Pasteurella haemolytica was demonstrated. It is concluded that the lentivirus may play an enhancing role in both viral and bacterial infections of sheep by compromising the host's cellular immune response.

INTRODUCTION

In jaagsiekte (ovine pulmonary adenomatosis), transformation of the alveolar epithelial lining is caused by the jaagsiekte retrovirus (JSRV), a member of the oncovirusae subfamily (Verwoerd, Tustin & Payne, 1985). The tumour can be transmitted efficiently by intratracheal inoculation of new-born lambs with the virus (Verwoerd, Williamson & De Villiers, 1980; Sharp, Angrey, Gray & Scott, 1983). However, considerable variation in the length of the period following infection, is characteristic of the transmission.

In many parts of the world jaagsiekte (JS) is closely associated with maedi, a chronic interstitial pneumonia caused by another retrovirus, maedi-visna virus (MVV), which belongs to the subfamily lentivirinae (Wanderla, 1971; Pallason, 1976; Snyder, De Martini, Ameghino & Caletti, 1983; Marksen, Spence & Dawson, 1983; Houwers & Terpstra, 1984). In South Africa a non-pathogenic lentivirus (SA-OMVV) was recently isolated and shown to be consistently present in jaagsiekte-infected lungs (Payne, York, De Villiers, Verwoerd, Qurat, Barben, Sauge & Vigne, 1986). This virus was found to be related to but not identical with maedi-visna virus (Quurat, Barban, Sauge, Vigne, Payne, York, De Villiers & Verwoerd, 1987).

The co-existence of the 2 retroviruses in a single animal has led to considerable speculation on the possibility of a synergism between them (Payne et al., 1986; De Martini, Rosadio, Sharp, Russell & Larmore, 1987). It has indeed been shown that whereas virus replication is restricted in experimental infections with visna virus (Gehalle, Ventura, Strawring & Haase, 1985), it could be transmitted efficiently to a natural case of jaagsiekte, with active replication of the lentivirus in the lungs (Dawson, Venables & Jenkins, 1985). As visna virus is known to replicate in vitro in macrophages (Narayan, Kennedy-Stokkopf, Sheffer, Griffin & Clements, 1983; Payne et al., 1986), one possible explanation for this observation is that the increase in the numbers of alveolar macrophages in jaagsiekte provides a larger reservoir of cells for the replication of lentivirus (De Martini et al., 1987). This increase is believed to be caused by a chemotactic factor secreted by JS-tumour cells (Myer, Verwoerd & Garnett, 1987 a). The in vitro interaction of these macrophages with other lymphoid cells results in the production of a factor which stimulates the growth of JS tumour cells, thus creating the possibility of further macrophage accumulation (Myer 1987; Myer, Verwoerd & Garnett, 1987 b).

An alternative explanation would be an immunosuppressive effect by JSRV, similar to that of Mason-Pfizer monkey virus to which it seems to be related (Sharp & Herring, 1983).

On the other hand, some lentiviruses are known to exert a powerful immunosuppressive effect. The best example of this is the human immunodeficiency (HIV-I) or AIDS virus (Popenia, Sarngadharan, Read & Gallo, 1984). It was therefore decided to investigate whether lentivirus-mediated immunosuppression plays a role in the etiology of jaagsiekte.

MATERIALS AND METHODS

Viruses

JSRV was obtained and stored as lung rinse pellets from experimentally produced jaagsiekte cases as previously described (Verwoerd, Payne, York & Myer, 1983). These pellets were shown to contain both JSRV and lentivirus (SA-OMVV). Lentivirus was isolated by culturing alveolar macrophages from experimental jaagsiekte cases for 1-2 weeks. Lung rinse fluid was centrifuged at low speed (400 x g for 30 min), the sediment resuspended in medium and the macrophages allowed to adsorb to the surface of plastic cell culture flasks for 120 min at 37°C. Medium was replaced at 3-day intervals and tested weekly for the presence of reverse transcriptase (RDP) activity (Verwoerd et al., 1983). Lentivirus positive medium was centrifuged (54 000 x g for 90 min) and the resulting pellet used directly, or for infection of sheep choroid plexus cells (Payne et al., 1986).

Infection of lambs

Experimental lambs were inoculated intratracheally within one week of birth with either SA-OMVV alone, or with pellets containing both JSRV and SA-OMVV. Virus concentrations were estimated by means of the RDP assay and adjusted to contain 1 x 10^6 RDP units in a 4.0 ml dose. The two groups of animals were kept under semi-isolated conditions to prevent cross-contamination.
IMMUNOSUPPRESSION CAUSED BY A LENTIVIRUS IN THE AETIOLOGY OF JAAGSIEKTE

Skin sensitization assay

The in vivo cellular immune response of experimental animals was tested by means of a delayed hypersensitivity skin test. After sensitizing the sheep with 10 mg of killed Mycobacterium avium in 5 ml of liquid paraffin injected intramuscularly, 0.1 ml of PPD of avian tuberculin was inoculated intradermally 6 weeks later. The thickness of folded skin was measured at 0 h, 48 h and 72 h using a Haupntner caliper. In the case of JS-sheep, the sensitization was started as soon as clinical symptoms of the disease were seen, in order to measure the animals’ cellular immune response at a comparable stage in the development of the tumour.

Isolation of monocytes

Monocytes were isolated from the peripheral blood of sheep essentially according to the method of Carlson & Kaneko (1973). Blood collected in 2 heparinized vacutainer tubes per sheep was centrifuged for 30 min at 1 000 × g. The buffy coats were pooled and resuspended in 4 ml of Dulbecco’s phosphate buffered saline (PBS). Remaining red blood cells were lysed by the addition of 20 ml of distilled water, followed by the restoration of isotonicity by adding 10 ml of PBS containing 2.7 % NaCl after 30 s of gentle mixing. The white blood cells were centrifuged (400 × g for 10 min), washed once in PBS and then resuspended in F-12 nutrient medium6. The monocytes were subsequently removed from the white blood cell fraction by adherence to a plastic substrate for 2 h at 37 °C. Fresh F-12 nutrient medium was added to these tissue culture flasks and the adherent monocytes were dislodged by vigorous shaking.

Thymidine incorporation assay

Monocyte suspensions were added to the upper compartments of Modified Boyden Chambers. Glass fibre membranes separated upper and lower compartments of the chambers, where the latter compartment contained F-12 nutrient medium with foetal calf serum serving as a normal, healthy sheep, as described in Myer, et al., 1987a, & al., 1987a, & al., 1987a, & al., 1987a. Monocytes were isolated from the peripheral blood of sheep essentially according to the method of Carlson & Kaneko (1973). Blood collected in 2 heparinized vacutainer tubes per sheep was centrifuged for 30 min at 1 000 × g. The buffy coats were pooled and resuspended in 4 ml of Dulbecco’s phosphate buffered saline (PBS). Remaining red blood cells were lysed by the addition of 20 ml of distilled water, followed by the restoration of isotonicity by adding 10 ml of PBS containing 2.7 % NaCl after 30 s of gentle mixing. The white blood cells were centrifuged (400 × g for 10 min), washed once in PBS and then resuspended in F-12 nutrient medium6. The monocytes were subsequently removed from the white blood cell fraction by adherence to a plastic substrate for 2 h at 37 °C. Fresh F-12 nutrient medium was added to these tissue culture flasks and the adherent monocytes were dislodged by vigorous shaking.

Chemotaxis assay

Assays for the chemotactic activity of various macrophage cultures were done as previously described (Myer et al., 1987a). Briefly, the chemoattractant used was either conditioned medium from 15.4 tumour cell cultures or N-formyl-L-methionyl-L-phenyl-alanine (N-FMett). A stock solution of 10-M N-FMett was diluted 1:25 in F-12 nutrient medium prior to use in the assay and arbitrarily referred to as 100 % N-FMett. Varying concentrations of this solution was added to the lower compartments of modified Boyden chambers, which were separated by 10 μm pore size polycarbonate membranes5 from the upper compartments containing 103 macrophages. After 3 h of incubation at 37 °C, the distance that the cells had migrated into each membrane was measured microscopically as described by Myer et al., 1987a.

Infection with Pasteurella haemolytica

A P. haemolytica Type 1 strain, originally isolated from a field case, was passaged twice in mice to increase virulence before inoculation into serum broth for overnight culture. It was then transferred to 200 ml of tryptose phosphate broth containing 0.5 % glucose. After incubating overnight at 37 °C on a rotary shaker, it was transferred to fresh tryptose-glucose broth in which it was grown for another 6 h. The bacteria were then harvested by centrifugation (800 × g for 60 min), washed and suspended in 10 ml of saline. Plate counts were performed to determine the concentration of viable organisms. Both lentivirus-infected and control animals were inoculated intratracheally with 1 × 1010 organisms in a volume of 10–20 ml, according to the size of the animal. Temperatures were taken daily for a period of 3 weeks and all animals observed for clinical signs of respiratory distress.

RESULTS

Cellular immune response in jaagsiekte-infected sheep

When jaagsiekte is transmitted to new-born lambs by intratracheal inoculation with pellets containing both JSRV and SA-OMVV, the latent period before clinical signs appear varies between 3 months and a year. In a few individuals no symptoms are seen after a year and only small, localized lesions are revealed by necropsy. In order to test whether suppression of the cell-mediated immunity (CMI) could be responsible for this variation in susceptibility, the delayed hypersensitivity response of each sheep was measured by means of a tuberculin skin test as described under MATERIALS AND METHODS. The 48 h differential measurements are shown in Fig. 1 as a function of the time of onset of jaagsiekte symptoms. The regression curve, fitted to the data according to Dunn (1977), clearly indicates a linear relationship, suggesting that there is a positive correlation between the latency period of clinical jaagsiekte and the CMI of the animal.

---

1 Strain 20485 local strain;—isolated from porcine lymph node
2 Produced at the Veterinary Research Institute, Onderstepoort, from M. avium strain 14, containing 25 000 tuberculin units/1 ml
3 F-12 nutrient medium: F-12 medium (Ham, 1965) supplied by High-veld Biological (Pty) Ltd, Northway, Sandon, Johannesburg, South Africa, and containing 20 % foetal calf (supplied by the same company), penicillin, streptomycin and nystatin
4 Amersham International, Ltd., Amersham, England
5 Sigma Chemical Company, St. Louis, USA
6 Nucleoco Corporation, Pleasanton, CA 94566-3294 USA
7 DIFCO
Immunosuppressive activity of the 2 viruses

The immunosuppression accompanying the development of jaagsiekte lesions could be due to either or both of the retroviruses. In Fig. 2, the average skin reactions are compared in 3 groups of sheep: normal control animals, sheep infected with lentivirus only and sheep infected with both lentivirus and JSRV. A significant suppression of the delayed hypersensitivity reaction is seen after lentivirus infection, with possibly an additional effect when JSRV also is present.

Monocyte involvement in the immunosuppression

The relatively mild nature of the immunosuppression detected in jaagsiekte-infected sheep suggested a different mechanism from that in AIDS, where inhibition of the T-helper lymphocyte function causes a dramatic inhibition of CMI (Wong, Staal & Gallo, 1983). Since it was known that the sheep lentiviruses have a natural tropism for cells of the macrophage lineage (Narayan et al., 1983), the ability of monocytes, isolated from JS-sheep, to synthesize DNA was compared with the skin test results of the same animals. A linear regression curve relating skin measurements to incorporation of tritiated thymidine (as a measure of DNA synthesis) is shown in Fig. 3. It suggests that reduced metabolic activity of monocytes may be responsible for the depressed cellular immune reaction, indirectly implicating the lentivirus in this process.

Effect of lentivirus replication on the chemotactic response of alveolar macrophages (AMs)

Replication of lentivirus has been shown to depend on the level of differentiation/maturatiopn of the monocyte/macrophage (Gendelman, Narayan, Kennedy-Stoskopf, Kennedy, Ghoibi, Clements, Stanley & Pezeshkpour, 1986). In order to assess the effects of lentivirus replication of their chemotactic activity, alveolar macrophages were collected from JS-infected sheep by lung lavage and cultured for 10 days in vitro. The clarified supernatants of these cultures were then tested for the presence of virus by means of reverse transcriptase and immunoblot assays as previously described (Payne et al., 1986). Two AM preparations positive for lentivirus production (LV+) and 2 negative preparations (LV−) were then assayed for chemotactic potential in response to N-F Met, a synthetic chemoattractant, as described under MATERIALS AND METHODS. The response of these in vivo infected AMs is shown in Fig. 4. Positive chemotaxis was observed in the 2 negative control cultures (C and D), whereas in the two LV(+) cultures (A and B) the response was inhibited and a higher level of chemokinesis (25 % values) was observed.

To confirm that this loss of chemotactic activity was caused by the in vitro activation of lentivirus replication, the experiment was repeated using AMs from normal, healthy sheep. Cultures of these AMs were subsequently infected in vitro with lentivirus. When virus production was detected by the RDP assay after 3 days, the cells were tested for their ability to respond chemotactically to tumour cell supernatant (Myer et al., 1987a). Fig. 5 B
IMMUNOSUPPRESSION CAUSED BY A LENTIVIRUS IN THE AETIOLOGY OF JAAGSIEKTE

FIG. 4 Effect of lentivirus replication on the chemotactic response of alveolar macrophages infected in vivo with lentivirus illustrates a loss of chemotaxis in infected cells compared to that of normal macrophages (Fig. 5 A).

These results suggest that lentivirus replication in macrophages inhibit their response to a chemotactic stimulus.

**TABLE 1** $^3$H-thymidine incorporation by peripheral blood monocytes from normal, resistant and JS-sheep

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Group average cpm</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4</td>
<td>1115</td>
<td>± 30</td>
</tr>
<tr>
<td>Resistant</td>
<td>4</td>
<td>1131</td>
<td>± 102</td>
</tr>
<tr>
<td>Jaagsiekte</td>
<td>6</td>
<td>695</td>
<td>± 99</td>
</tr>
</tbody>
</table>

Pasturella haemolytica infection as a test for immunosuppression

As controls, a group of 9 lambs were sensitized with *M. avium* 1 week after birth. Nine weeks later a tuberculin test was carried out, the results indicating a normal group average skin swelling of 9.0 mm (Table 2), which was lower than the 13 mm obtained for a larger group of adult animals (Fig. 2). When these sheep were inoculated 2 months later with $1 \times 10^7$ *P. haemolytica* organisms, one showed no symptoms at all, 7 had a transient fever reaction only, and one animal died with acute septicaemia.

Seven lambs were infected with $1 \times 10^6$ RDP units of lentivirus within one week after birth and sensitized with *M. avium* 4–9 weeks later. Their delayed hypersensitivity response was measured after 6 weeks. The results show considerable variation between the individual animals, one animal showing a normal skin swelling of 10.4 mm. The average for the others was $5.8 \pm 2.3$ mm (not shown in Table 2) which is below that of the controls of $9 \pm 2.9$ mm. A student’s t test according to Dunn (1977), between the 9 and 5.8 mm averages, gave a t value of 2.3 (13 degrees of freedom), corresponding to a 97.5% level of significant difference between the control and lentivirus-infected sheep. By oversight these lambs were immunized with pasteurella vaccine at 2 weeks of age. Nevertheless, when they were challenged at an average age of 7 months, with the same high dose of virulent organisms as the controls, the reactions were significantly stronger (Table 2). Two out of seven animals died from acute septicaemia, and one from pneumonia, while 4 showed clinical signs of respiratory distress (pneumonia) such as coughing dyspnoea and nasal discharge. These 4 sheep recovered clinically, while *P. haemolytica*, reisolated from the lungs of those animals that had died, was subsequently shown to be type 1, thereby confirming that the fatal infection was caused by the organisms inoculated.

**DISCUSSION**

The human and ovine lentiviruses are similar in morphology and morphogenesis and also share some homologous nucleotide sequences (Gonda, Wong-Staal, Gallo, Clements, Narayan & Gilden, 1985). For both groups the primary target cell seems to be the monocyte/macrophage. In the case of HIV most primary isolates from brain and lung tissues produce 10 to 100-fold more virus in mononuclear phagocytes than in lymphocytes. The virus appears to have a unique ability to acquire an affinity for T4 lymphocytes however, and the prototype HIV, which was selected for growth in T cells, has a 10 000-fold lower ability to infect macrophages than T-cells (Gartner, Markovits, Markovitz, Kaplan, Gallo & Popovic, 1986). At least part of the immunosuppressive effect of HIV is thought to be caused by depletion of T4-lymphocytes.

In contrast visna virus, the prototype ovine lentivirus, does not seem to be able to infect lymphocytes. In addition, virus replication in macrophages is severely restricted in vivo (Gendelman, Narayan, Molineaux, Clements & Ghotbi, 1985). This may be the reason why
immunosuppression has not been observed in maedi-visna and other related infections such as ovine progressive pneumonia (OPP) (Larsen, Hylleth & Krogrud, 1982; Haase, 1986). Narayan, Sheffer, Clements & Tennekoon (1985) have shown that visna virus causes an interferon-inducing factor to be produced on the surface of infected macrophages which is recognized by lymphocytes of ovine and caprine species. These lymphocytes, in turn, produce an interferon (non-glycosylated, 5,4-64 kilodalton protein that is resistant to PH 2.0 and heat treatment) that probably restricts viral replication in infected macrophages and is most likely an important mechanism for the persistence of the virus in the host which may develop a chronic immunosuppressive syndrome. In addition, the interferon also results in persistent expression of Ia antigen on the surface of infected macrophages (Kennedy, Narayan, Ghotbi, Hopkins, Gendelman & Clements, 1985).

Using a delayed type hypersensitivity assay, we found a significant immunosuppression in sheep co-infected with lentivirus and jaagsiekte retrovirus. The fact that lentivirus replication is stimulated in jaagsiekte lungs (Dawson et al., 1985) would lead one to speculate that it may also be responsible for the immunosuppression observed, but a direct contribution by the JSRV cannot be excluded in this system. We were able to demonstrate, however, that infection with lentivirus only, will also cause a significant suppression of the delayed type hypersensitivity reaction in sheep.

It was furthermore shown that inhibition of monocyte activity is involved in the suppression, suggesting a primary role for the lentivirus which has the monocye/macrophage as primary target cell. Further evidence for the involvement of ovine lentiviruses in cell-mediated immunity is the demonstration of decreased lymphocyte-generated interleukin-2 and decreased concanavalin-A-induced suppressor cell activities in natural cases of OPP (Ellis & De Martini, 1985).

Friedlander, Jahrling, Merrill & Tobey (1984) have demonstrated inhibition of mouse peritoneal macrophage DNA synthesis with Arenavirus Pinchinde, while Dengue virus infection causes a sharp decline in their Fc-receptor-mediated attachment and phagocytosis of opsonized sheep erythrocytes (Chaturvedi, Nagar & Mathur, 1983). The pathogenesis of certain viruses is limited to maturation of macrophages as well as to the differentiation of monocytes to macrophages. Lentiviruses have a tropism for cells of the macrophage lineage, and replication of the virus increases as monocytes mature to macrophages (Gendelman et al., 1986). A similar situation was found in our sheep that were infected with lentivirus, and in these animals where in vitro replication of the lentivirus was occurring in macrophage cultures, a concomitant inhibition of chemotactic potential was demonstrated in these cells (Fig. 4 & 5).

Insertion of retroviral glycoproteins within infected macrophage membranes is thought to interfere with the Fc-receptor-cytoskeletal system (Marcelletti & Furmanski, 1978). Macrophage function, such as tumour target cell destruction, chemotaxis, phagocytosis and bactericidal activities all require an intact Fc-receptor-cytoskeletal system. Cianciolo, Hunter, Silva & Haskill (1981) reported that a 15 kilodalton protein, isolated from human malignant lymphomas, rendered human monocytes immobile. They were able to neutralize this effect by adding three different murine monoclonal antibodies against p15(E) of type C retroviruses. The bulky, hydrophobic p15(E) protein is thought to block microtubule formation that is required for cell movement (Rojko & Olsen, 1984).

Influenza virus infection of alveolar macrophages reduces their antibacterial efficiency, which is associated with secondary infections in the lungs of infected hosts (Warshawer, Goldstein, Akers & Kim, 1977). In these sheep where macrophage chemotaxis might have been inhibited (assuming in vivo replication of the lentivirus), secondary complications, such as bacterial pneumonia, could be expected to have led to a more rapid death of the host than would have been caused only by the lung tumour.

Since it has proved impossible to obtain JSRV preparations free from lentivirus, we decided to use an independent non-viral challenge system as an additional test.
IMMUNOSUPPRESSION CAUSED BY A LENTIVIRUS IN THE AETIOLOGY OF JAAGSIEKTE

for immunosuppression. Pasteurella haemolytica is a common cause of pneumonia in ruminants but is difficult to induce experimentally in normal animals. Reproducible infection has only been achieved after pre-infection with bovine herpesvirus in calves (Hilwig, Songer & Reggardo, 1985) or Mycoplasma ovipneumonia in sheep (Gilmour, Jones, Rae & Quirie, 1986). We have isolated lentivirus infections and monomavirus infections from field cases of pneumonia. Pasteurella haemolytica in sheep (unpublished results), suggesting the possibility that the virus may play a role in the aetiology of this disease. The results reported in this paper support this contention. Despite the fact that the lentivirus-infected lambs were immunized with Pasteurella haemolytica vaccine, challenge with a high dose of organisms clearly showed that lentivirus-infected lambs were more susceptible than the controls. The severity of the symptoms in individual animals did not correlate well with the severity of immunosuppression as reflected by decreased skin swelling. Such a correlation can be found, however, when the averages for the groups are compared, suggesting that immunosuppression is the mechanism by which lentivirus enhances infection with P. haemolytica. The discrepancy may reflect inconsistencies in the delayed type hypersensitivity assay due to variable responses between individual sheep, or its ability to reflect which lymphoid cell component is involved in immunosuppression.

It can be concluded, therefore, that ovine lentiviruses cause a mild immunosuppression in sheep, mainly by affecting the monocyte/macrophage activity. The suppression of the cellular immune response in turn, predisposes the animal to secondary infections such as jaagsiekte and pasteurellosis.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the excellent technical assistance of Erika Stander, Suzette Olivier, S. Drake, Marianne Oosthuizen and Gottfried J. Schiele.

REFERENCES


