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The restricted IgG1 antibody response to maedi visna virus is seen following infection but not following immunization with recombinant *gag* protein

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SUMMARY

Maedi-visna (MVV) is a retrovirus of the subfamily lentivirinae which includes HIV, simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV). Infection of its natural host, the sheep, does not cause overt immunodeficiency, but rather a chronic inflammatory disease. However, subtle immunological changes following infection have been reported including a sheep IgG1 subclass-restricted MVV-neutralizing antibody. Here we demonstrate by Western blotting that there is no IgG2 serum antibody response to any MVV antigen after MVV infection, in contrast to infection with the parapox virus Orf, when serum IgG2 anti-Orf antibody is readily detected. By ELISA, the IgG1 antibody titres to Orf are higher than to MVV, but the minimum MVV serum antibody IgG1/IgG2 ratio is significantly raised compared with that for Orf virus antibody in the same sheep, indicating that the IgG2 defect in MVV infection cannot be accounted for by differences in the sensitivity of the Orf and MVV ELISA. Serum IgG2 anti-MVV *gag* p. 25 can be detected in both normal and MVV-infected sheep following immunization with purified recombinant MVV *gag* p 25 protein in Freund's complete adjuvant. The failure to make an IgG2 MVV-specific antibody indicates that immunological dysfunction can arise with macrophage tropic lentiviruses, and it may aid viral persistence.

Keywords antibody visna sheep lentivirus

INTRODUCTION

Maedi-visna virus (MVV) is a retrovirus of the subfamily lentivirinae which includes HIV, simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV) [1,2]. It naturally infects sheep, causing a slow progressive pathology after a long incubation period. The pathology is one of chronic inflammation which classically results in interstitial pneumonia in the lungs and demyelinating leukoencephalomyelitis in the brain.

The cellular receptor for MVV is unknown; it does not infect $CD4^+$ lymphocytes and is restricted in tropism to cells of the monocyte/macrophage lineage and possibly other antigen-presenting cells [3–5]. There is no gross immunodeficiency as is seen following HIV or SIV infection. There are, however, more subtle alterations in immune function in chronic MVV infection. Antibody responses to ovalbumin in Freund's complete adjuvant (FCA) are slightly reduced compared with uninfected controls (unpublished data), and there is an attenuated *in vivo* delayed hypersensitivity reaction to skin challenge with purified protein derivative (PPD) following bacille Calmette–Guérin (BCG) vaccination [6]. In addition, the serum antibody response to MVV has been reported to be restricted in IgG isotype, with purified IgG1 but not purified IgG2 anti-MVV antibody possessing neutralizing activity [7,8].

Ruminant IgG is comprised of two major subclasses, IgG1 and IgG2, although another minor isotype may exist, as four $C\gamma$ genes, one a pseudogene, have been identified in cattle [9,10]. IgG1 is the predominant immunoglobulin in sheep serum (2/3 of total serum IgG) and IgG2 is generally found at lower concentrations (1/3 of total serum IgG). Immunization of sheep with protein antigens normally generates antibody responses in both IgG isotypes. Carbohydrate antigens, pneumococcal polysaccharide or killed *Staphylococcus aureus* immunization elicit predominantly IgG1 and IgM antibodies

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[11]. A study on the influence of antigens and adjuvants on the production of interferon-gamma (IFN-γ) and IgG isotype in sheep found no evidence that the secretion of IFN-γ correlated with the production of antibody isotypes as is seen for IgG2a in the mouse [11,12].

IgG2 deficiency in cattle has been described in association with increased susceptibility to pyogenic bacterial infections [13] and has been shown to be important for protection against *Staph. aureus* mastitis in sheep [14]. Both IgG1 and IgG2 can activate sheep complement [15]. Studies using rosetting assays to measure binding of immunoglobulin-sensitized erythrocytes suggest the presence of at least two ruminant FcγR, one with high affinity for IgG2 which is highly expressed on neutrophils and macrophage/monocytes, and the other with high affinity for ruminant IgG1 which is highly expressed on alveolar macrophages and cultured monocytes and has recently been cloned [16–20]. Since the major effector cells of antibody-dependent cellular cytotoxicity (ADCC) in ruminants are reported to be neutrophils and monocyte/macrophages [21], IgG2 antibody is likely to play an important role in ADCC. The reported absence of an IgG2 antibody response to MVV may indicate a subtle defect in immunity to MVV in sheep by limiting ADCC against the virus-infected macrophage, which could have important implications for pathogenesis. We have therefore investigated this defect more fully by comparing the antibody response following infection with the retrovirus MVV with that with the parapox virus Orf [22]. By immunizing normal and MVV-infected sheep with recombinant MVV *gag* protein [23] we show that the failure of the IgG2 response to MVV is a failure of viral-infected sheep but not of viral antigen-challenged sheep.

MATERIALS AND METHODS

Animals

Adult Texel sheep naturally infected with MVV were obtained from a single flock. All animals used were seropositive by an agar gel immunodiffusion test. Control uninfected Texels were obtained from an accredited MVV-free flock. Four Finnish Landrace or Landrace Dorset crossed sheep were experimentally infected by s.c. injection of 10^6 $TCID_{50}$ infectious doses of MVV strain EV1 [24]. Almost all adult sheep have been exposed to Orf virus and have serum antibodies to Orf antigens, and these four sheep were no exception. These sheep were serially bled from the jugular vein pre- and post-infection over a period of 3 years and their heparin/plasma or serum stored at −20°C. Two naturally MVV-infected adult sheep and two control sheep were immunized subcutaneously with 35 μg of purified recombinant MVV *gag* p 25 in FCA and again in Freund's incomplete adjuvant (FIA), 2 weeks later. Serum samples were collected pre, 2 and 6·5 weeks post initial immunization.

Specific MVV or Orf antibody

Western blotting. MVV antigen was prepared from MVV-infected fibroblast cell cultures showing extensive cytopathic effects. Culture supernatants and infected cells were collected and pelleted by centrifugation (10 000 ***g***, 4°C, 16 h). The pelleted material was resuspended in PBS and then centrifuged at 120 000 ***g***, 4°C, 3 h, to concentrate and disrupt the virus/cell mix. The pellet was resuspended in PBS and stored at −80°C. Orf virus antigen was prepared by density gradient centrifugation from the scabs of Orf-infected sheep [25]. The virus antigen preparations were separated by discontinuous SDS–PAGE on 5–20% linear gradient gels. The proteins were transferred to nitrocellulose membranes (Hybond C; Amersham, Aylesbury, UK) using a semi-dry blotter (BioRad, Hemel Hempstead, UK) according to Khyse-Andersen [26]. The nitrocellulose membranes were blocked in PBS with 5% non-fat milk (Sainsbury plc) (blocking buffer) and stored at −20°C. Blots were incubated overnight at 4°C in either (for MVV) 1:10 dilution of serum from six sheep naturally infected with MVV, or (for Orf) 1:50 dilution of serum from three Orf-infected sheep. The immunoblots were developed with MoAb specific for sheep IgG1 (McM1), or IgG2 (McM3) kindly donated by Dr K. Beh [27] or normal mouse serum followed by alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (Sigma, Poole, UK), and finally developed with nitrotetrazolium blue (Sigma) + 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

ELISA. For the MVV ELISA, sucrose gradient-purified MVV [28] was coated at 1 μg/ml onto flexible Falcon PVC plates by overnight incubation at 4°C. For the Orf-specific ELISA, a 1% Nonidet NP40 extract of virus-infected cells was coated similarly at 3 μg/ml [25]. After washing in borate-buffered saline (BBS)/0·05% Tween 20, the wells were blocked by incubation with BBS/1 mg/ml bovine serum albumin (BSA; Sigma) for 1 h at 37°C. Doubling dilutions of plasma samples diluted in BBS/BSA/0·05% Tween 20 were incubated for 1 h at 37°C. Both ELISAs were developed identically with MoAb to sheep IgG1 (McM1) or IgG2 (McM3) diluted in BBS/BSA followed by horseradish peroxidase (HRP) rabbit anti-mouse immmunoglobulin previously absorbed against sheep IgG (Dako, High Wycombe, UK). Negative controls included uninfected cell extract coats and an irrelevant MoAb. All serum gave below twice background optical density (OD) in these controls. For each virus ELISA serum IgG1 and IgG2 end point titres (taken as twice no serum background OD) were calculated and the ratio of IgG1 to IgG2 antibody derived by dividing the two relevant end point titres. Since no MVV-specific IgG2 antibody was detected in any sheep at a starting serum dilution of 1:10, all MVV ratios are underestimates, as a value of 10 was used as the IgG2 anti-MVV titre.

Preparation of recombinant (r) MVV gag *p 25.* The expression of this major core protein, p 25, of MVV strain EV1 in yeast as a Ty-p25 fusion protein which self assembles to form virus-like particles that are purified by density gradient techniques has been previously described [23]. Rp25 was recovered by factor Xa cleavage from the Ty particles followed by centrifugation. Mock rp25 was prepared by omitting the enzymic cleavage before centrifugation. Both preparations contained similar trace yeast contaminants on SDS–PAGE analysis.

RESULTS

IgG1 and IgG2 antibody to MVV and Orf antigens following infection

MoAbs specific for sheep IgG1 and IgG2 have been used to detect the isotype of MVV-specific antibodies in six naturally MVV-infected adult sheep sera by the Western blot technique. The major MVV antigens are the *gag* proteins p 17 and

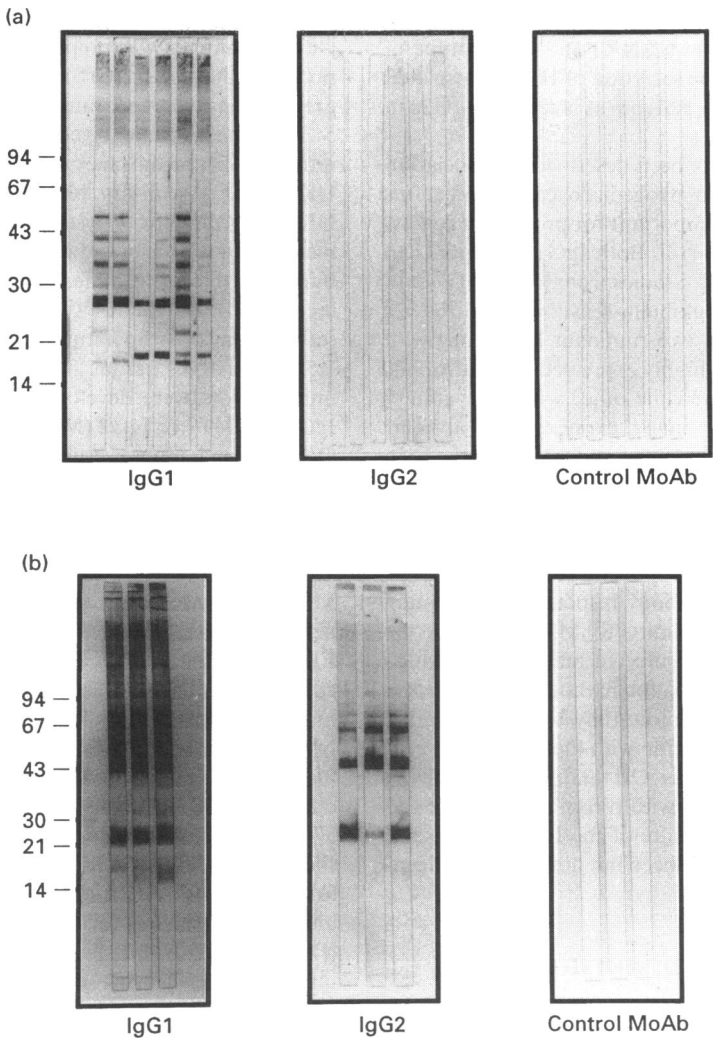

Fig. 1. Immunoblot analysis of the IgG1 and IgG2 antibody response to maedi visna virus (MVV) (a) compared with Orf virus (b). Western blots of MVV and Orf antigens were incubated in six naturally MVV-infected (a) or three Orf-infected sheep sera (b). Blots were developed with MoAb to IgG1 (McM1), IgG2 (McM3) or normal mouse serum as control, followed by alkaline phosphatase-conjugated anti-mouse immunoglobulin and chromogen.

p 25, together with the outer envelope (*env*) glycoprotein gp135 which is poorly retained during MVV antigen purification. Other virus-specific antigens may represent *gag* precursor proteins and/or *pol* gene products. Figure 1a shows the presence of IgG1 antibodies in all six sheep to *gag* p 25 and weakly to *env* gp135 and variably to a number of other virus antigens. There is no IgG2 antibody response to any MVV antigen.

For comparison, the serum antibody isotypes produced in response to Orf virus were analysed (Fig. 1b). Purified virions of Orf have been shown to contain at least 16 viral antigens recognized by infected sheep sera, with a polypeptide of 40–45 kD [29] being a major Orf virus protein. This protein is believed to be a component of the surface tubule-like structures on the virion [29]. Other viral proteins of mol. wt 16·5, 22·5, 25·5 and 64 kD are also believed to be components of the virion, but their function has not been identified.

Both IgG1 and IgG2 antibody can be clearly detected to several Orf antigens in Orf-infected sheep serum (Fig. 1b), indicating that the restricted IgG1 antibody response to MVV is not seen following natural Orf virus infection. Experimentally MVV-infected sheep serum also showed the complete absence of an IgG2 MVV-specific antibody response by Western blot analysis (data not shown).

For four sheep, the serum IgG1 and IgG2 antibody response to MVV was quantified by ELISA pre and 2–3 years post experimental MVV infection and compared with the IgG1 and IgG2 anti-Orf antibody titre analysed in the same sheep serum samples taken immediately before MVV infection (Fig. 2). In persistently infected MVV sheep serum the mean IgG1 MVV antibody titre was 615, with no detectable IgG2 antibody response to MVV (titre < 10). The same adult sheep sera show a high titre IgG1 and IgG2 response to Orf (mean titre IgG1 = 16 325; IgG2 = 1937) before MVV infection. IgG1 and IgG2 Orf antibody was also detected at high titre in the post-MVV-infected serum (data not shown). Specific pathogen-free lambs given a primary experimental Orf infection had developed both IgG1 (median 6400) and IgG2 (median 120) antibodies to Orf 7 weeks post-infection, but the

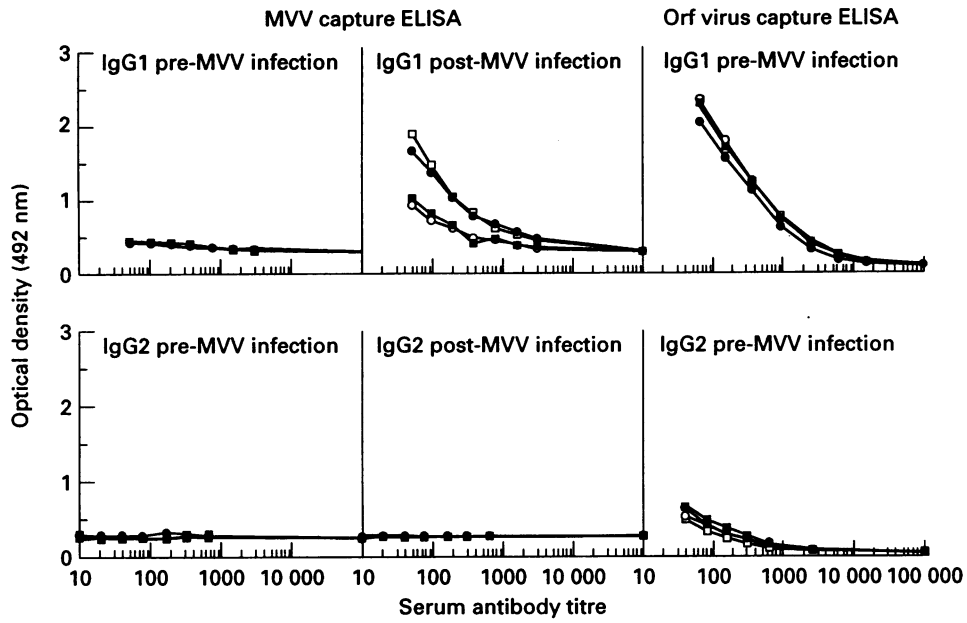

Fig. 2. Comparison of the IgG1 and IgG2 response to maedi visna virus (MVV) and Orf virus by capture ELISA in the serum of four sheep pre- or 2–3 years post-MVV EV1 experimental infection. Sheep serum were titrated onto MVV or Orf antigen-coated plates. The plates were developed with MoAb to IgG1 (McM1) or IgG2 (McM3) followed by horseradish peroxidase-conjugated anti-mouse immunoglobulin and OPD chromogen. ●, 848; ■, 865; □, 1540; ○, 1550.

IgG2 antibody titre in particular was much lower than in adult sheep serum (data not shown).

The sheep IgG2 antibody response to many antigens [9], including Orf virus described here, is lower than the IgG1 response, and reflects the total serum levels of these IgG isotypes. Since the IgG1 anti-MVV antibody titre was considerably lower than that to Orf, a low IgG2 anti-MVV response could go undetected by ELISA. To rule out this possible quantitative difference in IgG2 titres, the MVV and Orf virus-specific IgG1:IgG2 serum antibody ratios within identical sera from six sheep were compared (Fig. 3). All six sheep had been experimentally infected with MVV for at least 18 months, and the common occurence of Orf virus infections in sheep flocks had led us to assume that the sheep had been infected with Orf, probably repeatedly, for a considerable period before MVV infection [30]. In all six sheep, the IgG1:IgG2 ratio was higher for the MVV antibody response than for the Orf response, and there was a significant difference between these two ratios (P = 0·02) by Mann–Whitney non-parametric analysis. In addition, MVV infection for at least 18 months did not alter the Orf IgG1:IgG2 ratio significantly in these six sheep (data not shown). It therefore appears that there is a real defect in the IgG2 response to MVV following infection that is confined to the MVV antibody response and cannot be explained quantitatively.

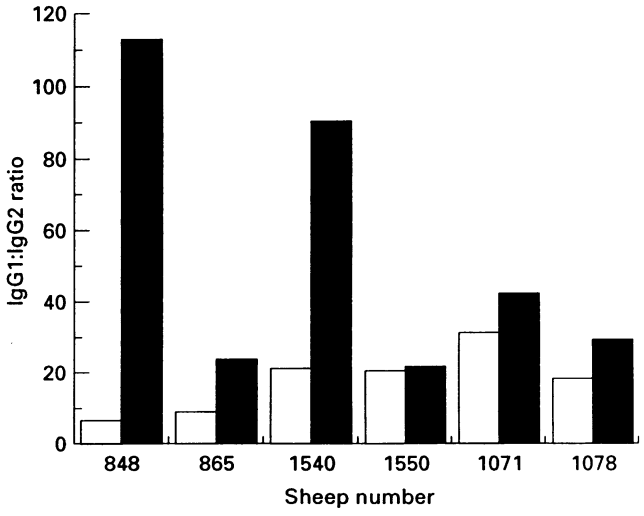

Fig. 3. The IgG1/IgG2 antibody titre ratio from capture ELISA for maedi visna virus (MVV) (■) or Orf virus (□) in serum from six sheep experimentally infected with MVV for at least 18 months.

IgG1 and IgG2 response to recombinant MVV gag *p 25 protein following immunization*

Immunization of two normal and two naturally MVV-infected sheep with purified recombinant *gag* p 25 protein was undertaken to try to raise an IgG2 anti-*gag* p 25 antibody response. Western blot of separated MVV antigens was used to compare the antibody response pre- and post-immunization in FCA (result for two sheep is shown in Fig. 4). The MVV-infected sheep showed a typical IgG1 restricted MVV antibody response before immunization (Fig. 4A (IgG1) and B (IgG2), track 1), whilst normal sheep had no anti-MVV antibody (Fig 4A,B, track 3). Following immunization with recombinant *gag* p 25, both the MVV-infected (track 2) and normal (track 4) sheep showed an IgG1 (Fig. 4A) and IgG2 (Fig. 4B) antibody response to viral *gag* p 25. Using sera pre- and post-immunization from the MVV-infected sheep, the intensity of the IgG1 anti-MVV p 25 band was greater post-immunization

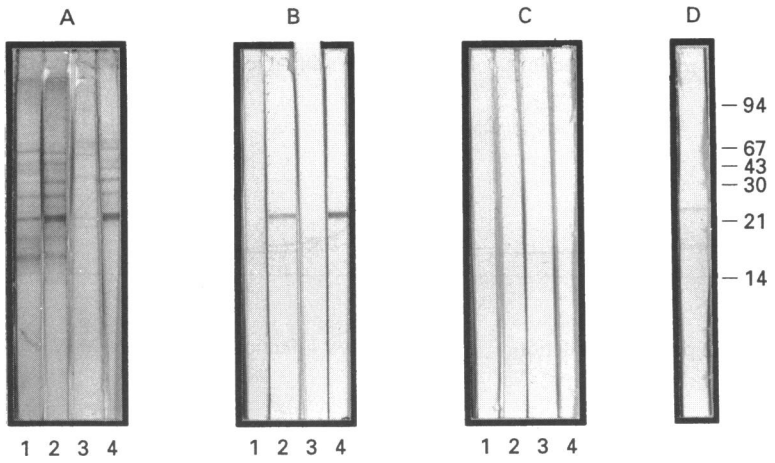

Fig. 4. The maedi visna virus (MVV)-specific antibody response in serum pre- and post-immunization with purified recombinant *gag* p 25 in Freund's complete adjuvant. A Western blot of MVV antigens was incubated in 1:100 dilution of serum from a naturally MVV-infected sheep pre- (track 1) and 6·5 weeks post- (track 2) immunization and from an uninfected control sheep serum pre- (track 3) and 2 weeks post- (track 4) immunization. Blots were developed with MoAb to IgG1 (McM1 at 1:2000) (A), IgG2 (McM3 at 1:500) (B), irrelevant MoAb (1:1000) as control (C), or MoAb to MVV rp25 (23) (D), followed by alkaline phosphatase-conjugated anti-mouse immunoglobulin and chromogen.

(Fig. 4A, track 2) than post-infection alone (Fig. 4A, track 1), suggesting that there had been an increased antibody response to p 25 and *gag* precursors following protein rp25 immunization. The other two sheep showed the same response on immunoblotting. Using a recombinant protein p 25 capture ELISA, the IgG1 antibody titres to purified *gag* p 25 increased in the MVV-infected sheep, but these ELISA data were hard to interpret, as the IgG1 antibody titres in a mock recombinant gag p 25 capture ELISA also increased possibly as a result of yeast-derived impurities in the recombinant protein preparations (data not shown). It is clear, however, from the immunoblot data that immunization with free MVV *gag* protein antigen overcame the specific defect in the IgG2 response to this antigen in MVV infection.

DISCUSSION

A restricted IgG1 antibody response to all MVV antigens which induce an IgG1 response has been demonstrated in sheep chronically infected with the lentivirus MVV. In contrast, the same sheep showed an IgG1 and IgG2 response to the parapox virus, Orf. However, the nature of these viral infections does vary. MVV causes a chronic slow virus infection with very little viral antigen detectable except within macrophages at clinically affected sites, e.g. bronchoalveolar lavage cells from infected lungs. Otherwise it is only possible to find rare MVV RNA-positive cells within draining lymph nodes, and only extremely rare MVV antigen-positive cells. MVV-infected sheep may survive for many years with no detectable clinical symptoms. In contrast, Orf virus, which is endemic amongst sheep and goats, causes a highly contagious eruptive skin condition typically with pustular lesions around the mouth and nares of lambs in which viral antigen can be readily detected. Infection normally resolves within 4–6 weeks, but protection against reinfection is not long lasting [25,30].

Using virus capture ELISA the IgG1 antibody titres to Orf were much higher than to MVV. This may be a real and important difference in the antibody response to these viruses, but it could result from differences in the sensitivity of the ELISA originating from the viral preparations used to coat the plates. The proportion within the concentrate of MVV antigens and in particular MVV *env* gp135 which is lost during purification [31] may be much lower than that of Orf. Despite possible differences in the sensitivity of these ELISAs, the minimal IgG1:IgG2 ratios were significantly increased for MVV antibody compared with Orf virus antibody. This indicates that there is a real defect in the IgG2 response to MVV in infected sheep compared with the IgG1 antibody titre. Affinity differences between the IgG1 and IgG2 anti-MVV response might allow IgG1 to mask a low IgG2 response. Purification of IgG1 and IgG2 from infected serum would reveal this. However, previous reports on the absence of an IgG2 MVV-neutralizing activity using immunoglobulin fractionation to reveal the defect [7,8] make this possibility unlikely. MVV-infected sheep when challenged with antigens such as ovalbumin and keyhole limpet haemocyanin (KLH) make both IgG1 and IgG2 antibodies in a similar ratio to uninfected sheep (P.B., unpublished results). In addition, total IgG2 serum levels are not significantly reduced in MVV-infected sheep (P.B., unpublished results) and therefore the IgG2 deficiency reported here appears to be confined to the specific MVV response.

Recombinant *gag* p 25 protein immunization in FCA (which contains heat-killed *Mycobacteria*) generated a typical IgG1 and IgG2 antibody response to a protein antigen in 2/2 normal sheep. In addition, an IgG2 response to *gag* p 25 appeared for the first time in 2/2 chronically infected sheep. The IgG1 anti-gag p 25 response appeared to have increased in comparison with preimmunization levels, unlike antibodies directed against other MVV antigens as detected by Western blotting. This is in contrast to a report that repeated i.v. immunization of two chronically caprine arthritis-encephalitis virus (CAEV)-infected goats with live virus in the presence of heat-killed *Mycobacterium tuberculosis* and mineral oil led to the appearance of neutralizing antibody to CAEV confined to the

IgG1 fraction following staphylococcal protein A Sepharose fractionation [32].

There are several possibilities for the origin of the isotype restriction in MVV-infected sheep reported here and in analogous CAEV-infected goats [33,34]. There is convincing evidence in the mouse that the IgG isotype of antibody to T lymphocyte-dependent antigens is influenced by the type of cytokine released by helper T cells and other accessory cells [35]. In other lentiviral infections there is some evidence that a type 2 cytokine response may predominate in late HIV infection and in a group of goats susceptible to CAEV arthritis with early clinical signs of synovitis [36] (Cheevers, personal communication). Similarly, activation of one type of T cell subset with a particular cytokine profile in MVV infection could explain this isotype restriction, but Emery *et al.* [11] found no evidence that sheep IgG1 and IgG2 responses correlated with IFN-γ levels (the classic type 1 cytokine). In addition, all sheep and goats with MVV or CAEV appear to show isotype-restricted virus antibody responses regardless of their clinical state, and many remain clinically unaffected for life. The very low level of MVV antigen during infection suggests that almost all antigen may be presented by infected cells, macrophages and possibly dendritic cells. The infected cell may have altered antigen-presenting cofactors, cytokines or surface molecules such as B7, which may influence B cell activation. Immunization with free protein would allow normal (uninfected cell) antigen presention. However, in the CAEV-infected goat, immunization with considerable quantities of virus antigens did not elicit an IgG2 response [32]. One difference between this failure and our success at inducing an IgG2 response was the use of whole virions rather than recombinant *gag* protein, respectively, as immunogens. Other MVV proteins have been shown to have immunosuppressive activity which might lead to this isotype defect [37].

There are possible pathogenic consequences of this isotype restriction. Plasma from MVV-infected sheep contain antibodies capable of binding specifically to MVV-infected cells and mediating complement-dependent lysis of these cells. However, these same plasma samples do not appear to mediate MVV-specific ADCC activity [38]. Neutrophils and monocyte/macrophages are reported to be important effector cells mediating ADCC in ruminants [21], and given that IgG2, but not IgG1, Fcγ receptors are present on neutrophils and fresh monocytes, it is possible that a potential MVV-clearance mechanism is inoperative. However, the significance of this *in vivo* is unclear, as complement C3 activation via IgG1 MVV-specific antibody bound to MVV-infected cells should allow adherence of both ADCC effector cell types to infected cells. Susceptibility to infectious disease can be influenced by only minor defects in host defence mechanisms, particularly if there are several, and the antibody isotype restriction in MVV infection may be an example of this, for there are also reports of altered macrophage activities including phagocytosis following MVV infection [39]. Studies on the origin of the restricted antibody response may reveal further mechanisms by which MVV manipulates and evades host immunity.

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