Inhibition of host peripheral blood mononuclear cell proliferation ex vivo by Rinderpest virus

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Rinderpest, or cattle plague, is caused by Rinderpest virus (RPV), which is related most closely to human Measles virus (MV), both being members of the genus Morbillivirus, a group of viruses known to have strong immunosuppressive effects in vitro and in vivo. Here, it was shown that peripheral blood mononuclear cells (PBMCs) isolated from cattle experimentally infected with either wild-type or vaccine strains of RPV impaired the proliferation of PBMCs derived from uninfected animals; however, in contrast to either mild or virulent strains of wild-type virus, the inhibition induced by the vaccine was both weak and transient. Flow-cytometric analysis of PBMCs obtained from cattle infected with different strains of RPV showed that the proportion of infected cells was virus dose-dependent and correlated with lymphoproliferative suppression.

INTRODUCTION

Rinderpest, or cattle plague, is caused by Rinderpest virus (RPV), a morbillivirus related most closely to human Measles virus (MV). MV was the first virus reported to perturb immune function (Pirquet, 1908) and, although MV-induced immunosuppression has been studied extensively, the exact molecular mechanism(s) remain unresolved. All morbillivirus infections appear to result in immunosuppression in the host (Heaney et al., 2002), thus allowing secondary, often bacterial, infections to flourish, increasing the mortality associated with morbillivirus infection. Many mechanisms have been proposed to account for MV-induced immunosuppression, including the inhibition of alpha/beta interferon production, suppression of the inflammatory response, altered cytokine profiles, direct infection and subsequent destruction of leukocytes, inhibition of immunoglobulin synthesis and cell-cycle arrest after direct contact with viral glycoproteins, all aspects of which were recently reviewed by Schneider-Schaulies et al. (2001) and references therein. In an in vitro system, Schlander et al. (1996) showed that mitogen-stimulated proliferation of naïve peripheral blood mononuclear cells (PBMCs), known as responder cells (RCs), was impaired after co-cultivation with MV-infected, UV-irradiated PBMCs, known as presenter cells (PCs). This phenomenon was observed even at low PC:RC ratios or when RCs were co-cultured with UV-inactivated virus. The effect was abolished completely when the two cell populations were separated physically by a porous membrane, indicating that soluble factors were not responsible for immunosuppression in this model system (Schlander et al., 1996).

We demonstrated previously that UV-inactivated virus of all members of the genus Morbillivirus inhibited the proliferation of lymphoid cells similarly in culture. In the case of RPV, as for MV, direct contact of the two cell populations is essential for this phenomenon to occur and the effect is mediated through co-expression of the virus glycoproteins on the PC surface. Virus preparations from both wild-type and vaccine strains of RPV were shown to inhibit the proliferation of mitogen-stimulated bovine PBMCs in vitro (Heaney et al., 2002). Natural or experimental infections in cattle are clearly immunosuppressive, with virulent strains inducing severe destruction of cells in the lymphoid organs and a profound leukopenia in the infected hosts. Virus antigen can be detected in sites of lesions in the lymphatic system, and lymphodepletion is observed in the lymph nodes and Peyer’s patches (Wohlesin et al., 1993, 1995). RPV also induces apoptosis of infected leukocytes (Stolte et al., 2002). It has also been suggested that RPV vaccination could cause immunosuppression in the host (Jeggo et al., 1987; Stevenson et al., 1999). However, Lund et al. (2000) could find no evidence of immune suppression in cattle vaccinated with the RBOK vaccine strain, the most commonly used vaccine against this disease, in contrast to the profound suppression observed following experimental wild-type infection. Vaccination induced a strong T-cell response to the major virus structural proteins investigated.

Here we have shown, by using an ex vivo experimental system based on the in vitro proliferation assay described previously, that both wild-type and vaccine strains of RPV
impart the proliferation of host PBMCs, but that with the vaccine strain, in contrast to either mild or virulent strains of wild-type virus, the extent of the proliferation inhibition induced is both transient and weak.

METHODS

**Virus strains.** Three wild-type isolates of RPV were used: Saudi/81 (Taylor, 1986), obtained as freeze-dried spleen extract from a previously infected animal; Kenya/kudu/96 (Kock et al., 1999), passaged twice in B95a cells and Kabete ‘O’, also passaged twice in B95a cells. The TCID_{50} of wild-type RPV stocks was determined in B95a cells by using the method of Reed & Muench (1938). Two vaccine strains of RPV were used. Stocks of the Plowright vaccine strain (RBOK) (Plowright & Ferris, 1962) and a recombinant version of the vaccine strain that was engineered to express the P1 protein of *Foot-and-mouth disease virus* (FMDV) (RPV–P1; P. Walsh & T. Barrett, unpublished) were grown and titrated in Vero cells.

**Animal vaccinations and challenge.** All experiments were performed in accordance with animal-welfare legislation and were part of the programme for the development of new-generation RPV vaccines (Barrett et al., 2003). Groups of either two or four Friesian calves (6–12 months) were inoculated subcutaneously in the pre-scapular region with 1 ml suitably diluted virus. Unless otherwise stated, each virus dose was 10^{6} TCID_{50}. Twenty-eight days post-vaccination, experimental animals were challenged with virulent virus. Throughout the experiments, all animals were examined daily for clinical signs of disease and rectal temperatures were recorded.

**PBMC isolation.** Bovine PBMCs were isolated from heparinized blood samples (20 ml) collected at regular intervals throughout the duration of each experiment. Blood samples were centrifuged at 400 g at 4 °C for 10 min to obtain a buffy-coat layer. These cells were collected, diluted 1:2 in PBS and centrifuged over Histopaque 1083 (Sigma) for 30 min at 800 g at 18 °C to purify the PBMCs. Cells collected from the interface were diluted at least 1:2 and pelleted by centrifugation at 400 g for 10 min at 4 °C. The cells were resuspended in 2 ml PBS and 10 ml water was added, followed almost immediately by the addition of 10 ml 2× Eagle’s medium to lyse contaminating erythrocytes. PBMCs were washed three times with 10 ml PBS and then resuspended (1×10^{5} ml^{-1}) in RPMI 1640 (Gibco) containing 10% fetal calf serum (FCS; First Direct).

**Ex vivo proliferation assay.** *Ex vivo* proliferation assays were performed by using a system adapted from Schlender et al. (1996). PC populations were generated by isolating PBMCs from vaccinated or challenged animals at regular intervals over a 2-week period. PCs were generated from uninoculated animals housed in a separate animal unit. PCs were UV-inactivated and mixed, in decreasing ratios, with PCs (1×10^{7}) that had been stimulated with phytohaemagglutinin (PHA) (1 μg per 100 μl), both in a volume of 100 μl. The two cell populations were incubated for 72 h at 37 °C in an atmosphere of 5% CO_{2} before the proliferative response of the PCs was determined by using the MTT assay (see below).

**MTT assay.** MTT assays were performed as described previously (Heaney et al., 2002). In brief, 10 μl MTT (5 mg ml^{-1}; Sigma) was added to each assay well and incubated for 4 h, protected from the light. The formazan crystals produced were dissolved by resuspension in 100 μl lysis buffer [20% (w/v) SDS; 50% (v/v) dimethylformamide] and the A_{550} was measured by using a Labsystems Multiscan Plus ELISA reader.

**Flow cytometry.** Isolated PBMCs were washed with PBSA (PBS with 0.01% sodium azide and 1% FCS) prior to single-colour fluorescence-activated cell-sorting (FACS) analysis by using polyclonal rabbit anti-RPV hyperimmune serum. Cells were fixed with 3% paraformaldehyde and washed three times with PBSA before incubation with 25 μl primary antibody (diluted 1:1000) at 4 °C for 30 min in a Costar 96-well flat-bottomed plate (at least 1×10^{5} cells per well). Cells were washed with 100 μl PBSA, centrifuged at 400 g and labelled with 25 μl of the appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody (diluted 1:1000; Molecular Probes) for 30 min at 4 °C. Cells were again washed and resuspended in 200 μl PBSA before analysis by FACS (Becton Dickinson) using CellQuest software.

RESULTS

**Proportion of infected PBMCs following RPV infection**

Cattle were inoculated subcutaneously with the standard dose (10^{4} TCID_{50}) of the highly virulent recombinant Kabete ‘O’ strain of RPV (animals UN 73 and UN 74), with the standard (animals UG 63 and UG 64) or a 100-fold higher dose (animals UG 61 and UG 62) of a virus isolated from wildlife in Kenya (Kenya/kudu/96), or with the standard dose of the tissue culture-attenuated RBOK vaccine strain of RPV (animals UU 04 and UU 05). The kudu virus is known to be less pathogenic in domestic cattle than the Saudi/81 or Kabete ‘O’ wild-type RPV strains (Barrett et al., 1998; Kock et al., 1999; Taylor, 1986). PBMCs isolated from these cattle were fixed with 3% paraformaldehyde and labelled with rabbit polyclonal anti-RPV hyperimmune serum followed by goat anti-rabbit FITC-conjugated secondary antibody. FACS analysis revealed the proportion of the total population of PBMCs that were RPV-positive from each animal on various days following RPV infection. Animals that received the virulent recombinant Kabete ‘O’ RPV strain had a higher proportion of infected PBMCs (over 30%) from day 2 post-infection compared with animals infected with a similar dose (10^{4} TCID_{50}) of the less-virulent Kenya/Kudu/96 RPV strain (maximum 20%). The number of infected cells remained high following RPV Kabete ‘O’ inoculation, decreasing slightly towards the end of the test period (day 8) when the animals were euthanized due to the onset of severe clinical disease. Animals inoculated with the same dose of the less-pathogenic kudu strain showed a lower and variable number of infected cells, whilst animals that received a higher dose (10^{6} TCID_{50}) of this virus had a higher proportion of infected PBMCs (maximum 32%) at 2 days post-infection than the animals that received a lower kudu virus dose (10^{5} TCID_{50}). In all four animals infected with the mild Kenya/kudu/96 strain, there was a slight increase in the proportion of infected cells at days 9 and 12 post-infection, indicating a biphasic replication of the virus *in vivo*. Animals that received the higher dose of Kenya/kudu/96 virus only showed a transient pyrexia and slight lacrimation. Animals that received 10^{4} TCID_{50} of the RBOK vaccine had no more than 15% virus-positive PBMCs, which peaked after 2 days and then gradually decreased to pre-vaccination levels over the 14 day period of observation (Fig. 1). This was a marked
reduction in the proportion of infected cells compared with the virulent infection.

**Inhibition of PBMC proliferation by contact with PBMCs from cattle following RPV vaccination**

PBMCs were obtained from the RPV-P1-infected animals at various times after infection, UV-irradiated to generate a PC population and mixed in decreasing proportions (as indicated in the figures) with PHA-stimulated PBMCs derived from uninfected cattle, the RC population. After a 72 h co-culture period, inhibition of proliferation of RCs was determined by using the MTT assay. PCs inhibited the proliferation of RCs from an uninfected animal with peak inhibition of 45–50% observed on day 2 at a PC:RC ratio of 1:1. Thereafter, the level of suppression decreased and background levels were reached by day 9 post-vaccination (Fig. 2).
Inhibition of PBMC proliferation by contact with PBMCs from cattle following wild-type RPV infections

PC populations were also generated from cattle infected with wild-type strains of RPV. Proliferation of the mitogen-stimulated RC population was inhibited on co-culture with PCs from animals infected with the virulent Saudi/81 RPV strain (animals TX 21 and TX 24) as early as 2 days post-infection. Inhibition was dose-dependent, decreasing with decreasing PC : RC ratio, and remained high until day 8 post-infection, when the animals showed significant clinical disease and were euthanized. In one animal (TX 21), inhibition of proliferation decreased slightly on day 8 post-infection, but had increased again by day 9. Proliferation of RCs was also inhibited by PCs derived from all experimental animals infected with the mild strain of RPV (Kenya/kudu/96), both those that received the high dose (animals UG 61 and UG 62) and those that received the standard dose (animals UG 63 and UG 64) of the virus. However, at day 2 post-infection, the levels of inhibition observed were lower than those observed with PCs prepared from animals infected with the virulent virus, approximately 45–50 % at a PC : RC ratio of 1 : 1 for the high dose and between 30 and 40 % for the standard dose infection compared with 70–90 % for the Saudi/81 strain. Thereafter, inhibition levels decreased and returned to background levels by day 7, animal UG 61 being the exception. Subsequently, a slight increase in RC proliferation inhibition was observed at days 9 and 12, indicating biphasic replication of the kudu virus strain in the experimental animals (Fig. 3).

Inhibition of PBMC proliferation by contact with PBMCs from cattle following RPV vaccination and subsequent challenge

To determine whether the tissue culture-attenuated RPV vaccine could induce immunosuppression in the host, four cattle were vaccinated subcutaneously with $10^4$ TCID$_{50}$ of the RBOK strain of RPV. As before, a PC population was generated by UV-irradiating PBMCs derived from the vaccinated cattle over a 2-week period. PCs were co-cultured with the RC population for 72 h before the RC proliferative response was determined by using the MTT assay. At 2 days post-vaccination, PCs from all four vaccinated cattle (animals UB 84–87) inhibited the proliferative response of RCs by between 30 and 50 %. By day 7 post-vaccination, the immunosuppressive effect of the PCs was abolished in three out of four cases, the exception being UB 86. Proliferation inhibition was again observed at day 12 post-vaccination in animals UB 86 and UB 87 (Fig. 4). By day 14, PCs from all animals failed to inhibit RC proliferation.

One month after vaccination, cattle were challenged with the virulent Saudi/81 strain of RPV ($10^4$ TCID$_{50}$). All four animals tested showed complete protection, exhibiting no signs of clinical disease over the 2-week period of observation. Although they had developed protective immunity, PCs isolated from cattle post-challenge exhibited a slight inhibitory effect on the RC proliferative response. In one case (animal UB 84), the inhibitory effect was demonstrated at day 2 and persisted until day 10 post-virus challenge, but at low levels of < 30 %. In two animals (UB 85 and UB 86), the inhibitory effect on RC proliferation was more transient, beginning on day 5 post-challenge and lasting to day 9. One animal (UB 87) showedonly a low level of proliferation inhibition on day 5 and was normal thereafter (Fig. 4).

DISCUSSION

We have shown that PCs derived from either vaccinated cattle or cattle infected with wild-type strains of RPV can inhibit the ex vivo mitogen stimulation of uninfected heterologous bovine PBMCs. This indicates that contact-mediated suppression is relevant in vivo. The inhibition of RC proliferation observed was greatest in the case of the virulent strain (Saudi/81), but relatively high levels were also observed when the PCs were derived from cattle infected with a milder strain of RPV (Kenya/kudu/96). The increased levels of immunosuppression induced following infection with a virulent strain of RPV may account, at least in part, for the increased virus-induced pathogenesis in animals.
infected with such strains. Other factors, such as the rate of replication in different tissues and altered cell tropisms, may also play important roles in determining pathogenic potential, an area that has not yet been well explored, although altered replication rates rather than altered cell tropism is more likely (Scott, 1990; Wohlsein et al., 1993).

Flow-cytometric analysis of the PCs obtained from animals infected with the kudu virus (Kenya/kudu/96) revealed that the percentage of infected cells was virus dose-dependent: a greater proportion of the PBMCs were initially infected in the cattle that received the high dose compared with cattle infected with the lower dose. However, this difference diminished later in infection. The proportion of infected PBMCs over the 2-week test period was found to correlate with the ability of the PCs derived from each experimental animal to suppress RCs. The apparently higher production of virus antigen in tissues from animals infected with more virulent strains of the virus (Wamwayi et al., 1995; Wohlsein et al., 1994; Rey Nores & McCullough, 1996) is consistent with a greater number of PCs expressing the F and H glycoproteins on their surface being an important factor in governing RC suppression levels in vivo as well as in vitro. That both these proteins are required for contact-mediated inhibition of proliferation of bovine PBMCs has been shown by using recombinant viruses expressing these proteins (Heaney et al., 2002). The ability of RPV to infect different subsets of PBMCs has been well documented. Unstimulated macrophages are infectable; however, both stimulated B and T cells can be infected with RPV (Rey Nores & McCullough, 1996, 1997). Therefore, it would be interesting in future experiments to determine which subset(s) are affected by contact-mediated inhibition.

In the majority of cases, the RPV-induced suppression, seen with infections by both mild and virulent strains, was found to be biphasic. The initial peak around day 2 post-infection was generally followed by a dip and a second increase between days 6 and 12, depending on the RPV strain concerned. In the case of mild RPV virus infection (Kenya/kudu/96), the observed second increase in RC proliferation inhibition was found to correspond to a second increase in the proportion of RPV antigen-positive circulating PBMCs. The second phase of immunosuppression may correlate with the second viraemia, which has been observed for MV, following extensive replication in the lymph nodes (Griffin & Bellini, 1996). It is clear that PBMCs derived from animals with wild-type RPV infections, and even those inoculated with the vaccine strain, can clearly arrest mitogen-stimulated proliferation of uninfected PBMCs ex vivo, albeit to a much lesser extent, with the mild and vaccine strains.

In a previous study on the immunosuppressive effects of RPV vaccination, Lund et al. (2000) could find no evidence of impairment of T-cell responsiveness, either directly after vaccination or on subsequent challenge with a virulent RPV.
strain. This indicated that vaccination was not followed by a general immunosuppression. However, in that study, the effect of the vaccine on mitogen stimulation was only determined from 7 days after its administration, when the early, and very mild, suppressive effects shown in the present study would probably have disappeared. Similarly, on subsequent challenge with a highly virulent RPV strain, only very low levels of RC proliferation inhibition were observed in the present study and the onset was delayed until day 5 post-challenge in three out of four animals. We conclude that this observed suppressive effect on RC proliferation indicates some replication of the challenge virus in vaccinated animals. By day 10, the suppressive effect was abolished in all cases; thus, these results are in agreement with previous reports and show that RBOK vaccination not only fully protects animals against clinical disease, but also prevents the severe immunosuppressive effects of virulent RPV and hence is unlikely to allow opportunistic bacterial, or other pathogen, superinfections to flourish. Immunosuppression, however, is a complex and multifaceted process and other mechanisms of suppression need to be studied in vivo, for which the RPV–cattle virus–host system is an excellent non-model experimental system.

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![Fig. 4. Effect of co-culture of PBMCs from RPV RBOK-vaccinated and Saudi/81-challenged cattle with uninfected PBMCs. Four cattle (UB 84–87) were vaccinated subcutaneously with $10^4$ TCID$_{50}$ of the vaccine RBOK strain of RPV and challenged 28 days later with the Saudi/81 virulent strain of RPV. PBMCs were obtained at intervals over a 2-week period following vaccination and challenge, UV-irradiated and their effect on RC proliferation was determined as described in Fig. 2. Left-hand panels show the RC proliferation inhibition seen following vaccination and right-hand panels show the inhibition following challenge.](3354)
REFERENCES


