Rinderpest virus isolates of different virulence vary in their capacity to infect bovine monocytes and macrophages

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Introduction

Rinderpest virus (RPV) is a member of the genus Morbillivirus of the family Paramyxoviridae, and is closely related to measles virus (MV). Despite the fact that there is only one serotype of RPV, different isolates can exhibit different virulence in the same host species. Virulent RPV causes an acute febrile illness characterized by sharp fever, erosive stomatitis, gastroenteritis, dehydration and leukopenia (Scott, 1990). At present, RPV strains circulating in East Africa cause mild to moderately severe clinical disease in cattle (Wafula & Kariuki, 1987; Wamwayi et al., 1992); isolates recovered from animals in the Middle East are highly virulent and cause mortality exceeding 80–90% (Taylor, 1986). Monoclonal antibodies (MAbs) have been used to delineate the antigenic relationship between and within morbilliviruses (Sheshberadaran et al., 1986; McCullough et al., 1986, 1991; Libeau & Lefèvre, 1990). Analysis of the nucleotide sequence of different isolates provided evidence for different lineages of RPV, reflecting their geographical distribution (Barret et al., 1993; Chamberlain et al., 1993). However, the factors which determine the variation in virulence remain unknown, and have not been related to a single event.

Highly virulent isolates would appear to be capable of replicating more rapidly, which would result in rapid progression of the disease associated with extensive destruction of tissues (Plowright, 1964; Brown & Torres, 1994; Wohlsein et al., 1993, 1995). Low virulence strains probably replicate more slowly, resulting in their viral antigen being less extensively distributed through different organs and tissues. Such characteristics may account for the milder clinical disease and lower mortality observed with the low virulent compared with highly virulent strains of RPV (Taylor & Plowright, 1965; Wohlsein et al., 1995).

Both mild and virulent strains of RPV share essentially the same tissue tropism in vivo – epitheliotropism and lymphotropism (Wohlsein et al., 1995). In contrast, the avirulent RBOK vaccine strain is strictly lymphotropic (Taylor & Plowright, 1965). Within lymphoid organs, lymphocytes in the B and T cell areas seem to be permissive for both mild and highly virulent strains of RPV (Plowright, 1964; Taylor & Plowright,
1965; Tajima & Ushijima, 1971; Wohlsein et al., 1995), and a profound leukopenia accompanies the acute phase of virus infection. This is largely due to lymphopenia, but the precise mechanism of cell death is still unknown.

In vitro, bovine leukocytes have been infected with virulent and lapinized strains of RPV (Tokuda et al., 1962; Pigouey et al., 1967; Rossiter & Wardley, 1985), although the precise leukocyte target for infection was not clearly defined. Recent work employing MAbs specific for different leukocyte sub-populations demonstrated that bovine blood monocytes and monocyte-derived macrophages are the main leukocyte target for RPV (Rey Nores et al., 1995). They serve as host for a relatively slow but productive infection. Efficient infection of lymphocytes can only be achieved after activation with mitogens such as Concanavalin A or pokeweed mitogen; intercelular communication with endothelial cells can also increase the susceptibility of lymphocytes to RPV (Rey Nores & McCullough, 1996).

The present study was designed to make use of these observations on in vitro infection of monocytes with reference to variations in RPV isolate virulence. As to the objective, this was to determine if a relationship existed between the reported in vivo virulence of a particular isolate and its characteristics of infection in monocytes. Three strains of RPV with different in vivo virulence were employed. Their relative capacity to infect monocytes and monocyte-derived macrophages, induce morphological changes in the infected cells and produce progeny virus was examined.

Methods

**Cell and viruses.** African green monkey kidney (Vero) cells were obtained from the ATCC. Vero cells were grown in minimal essential medium (Gibco) supplemented with 10% fetal calf serum. These cells were used to propagate the following three RPV isolates: (i) the vaccine strain, RPV-RBOK, derived from the 'O' Kabete strain of RPV (Rey Nores & McCullough, 1996); (ii) RPV-Egypt, isolated in Egypt in 1984 during an outbreak that caused minimal mortality, and considered a mild isolate (Taylor, 1986); (iii) RPV-Saudi, isolated in Saudi Arabia during an outbreak of rinderpest in the early 1980s that caused nearly 100% mortality in cattle and considered to be a highly virulent isolate (Taylor, 1986). RPV-Egypt and RPV-Saudi were used at low in vitro passage. Stock preparations of the three viruses have the following passage history: Saudi BK3/MDKBK1/V5; Egypt V5, RBOK BK98/V4 (BK stands for primary calf kidney cultures, MDBK for Madin–Darby bovine kidney and V for Vero). All viruses were obtained from the Institute for Animal Health, Pirbright, UK, where their relative virulence had been characterized.

**Peripheral blood mononuclear cells.** Blood was obtained from two blood-donor calves by jugular venipuncture and collected into Alsever’s solution. Peripheral blood mononuclear cells (PBMC) were separated from the Buffy coat fraction by centrifugation at room temperature through endotoxin-free Ficoll–Hypaque (Pharmacia) at 1000 g for 30 min. Cells were washed three times (250 g, 10 min) with phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS-A). Culturing was in tissue culture flasks or tissue culture 12-well plates (Costar) in a 37 °C CO₂ incubator at 1–2.5 × 10⁶ cells/ml in phenol red-free Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% non-mitogenic newborn calf serum (selected to support the growth of bovine PBMC without being mitogenic to the cells), 1% bovine serum albumin (BSA; complete medium). Monocyte and monocyte-derived macrophage cultures were obtained from PBMC which had been incubated for 24 h or 7 days, respectively, in plastic culture flasks, at which time the non-adherent cells were removed by repeated washing with warm PBS (Rey Nores et al., 1995). The remaining adherent cells were shown to be > 85% monocytes by staining with an anti-CD14 MAb.

**Monoclonal antibodies.** The following MAbs were used: IVB2-4 (Libeau & Lefèvre, 1990), specific for the nucleoprotein (N) of RPV; C1 (Anderson & Mackay, 1994), specific for the glycoprotein (H) of RPV; PT58A (anti-BoLA class I), W6/32 (anti-HLA class I), cross-reacting with BOLA (Bensaid et al., 1988), H42A and TH16B (anti-BoLA class II), BAT75A (anti-bovine CD11a/18 (LFA-1)), all obtained from VMRD (Pullman, USA). The isotype controls were UPC-10 and MOPC21 (Sigma).

**Flow cytometry.** Monocytes and monocyte-derived macrophages were removed from the surface of the tissue culture flasks by incubation with 5 mM EDTA in PBS-A for 15–30 min at 4 °C, and washed once in CellWASH (Becton Dickinson) before staining. For immunofluorescence labelling, 2 × 10⁵ cells were first incubated with one of the following MAbs (C1, PT58A, W6/32, H42A or the corresponding isotype controls) for 30 min on ice. After two washes with CellWASH, FITC-labelled Fab'², rabbit anti-mouse immunoglobulins (Dako) were added and incubated for 30 min on ice, followed by further washing. Fluorescence staining was determined by flow cytometry using a FACScan flow cytometer (Becton Dickinson) and the Lysis II software program. At least 5000 cells per sample were acquired, and dead cells plus debris excluded from the analysis by propidium iodide labelling and gating around live cells.

**Immunofluorescence and light microscopy.** Monocytes and monocyte-derived macrophages were cultured in 12-well tissue culture plates and infected as described above. At different times post-infection (p.i.), cells were washed once with PBS and fixed in 4% paraformaldehyde for 15 min followed by washing in PBS. Cells were permeabilized with 0.1% Triton X-100 detergent (Merck) for 5 min, followed by washing before staining with MAb IVB2-4 against the N protein of the virus. After 30 min incubation at 37 °C in a humid chamber, plates were washed (3 × 5 min) and stained with Fab'(2) rabbit anti-mouse immunoglobulins (Dako) for 30 min at 37 °C in a humid chamber.

For cell morphology studies, cultures were fixed and stained with Diff-Quick (Boehringer). Cells were visualized with a Leica microscope equipped for immunofluorescence.

**Virus infectivity assay.** Cell and supernatant fractions from the infected and mock-infected cultures were harvested at different times p.i.
Results

Susceptibility of bovine monocytes and macrophages to in vitro infection with different isolates of RPV

The susceptibility of bovine monocytes to infection by RPV isolates of different in vivo virulence was studied. Virus H glycoprotein expression, determined using flow cytometry, in replicate cultures infected with RPV-Saudi (highly virulent), RPV-Egypt (mildly virulent) or the vaccine strain RPV-RBOK is shown in Fig. 1. At day 1 p.i., it was not possible to identify clearly infected cells in any of the cultures. By day 3 p.i., RPV-Saudi had infected and replicated in more monocytic cells than the other two isolates, with the RPV-RBOK culture showing the lowest percentage value for infected cells. At 6 days p.i., this difference in the efficiency of the three isolates to infect and replicate in monocytic cells was more obvious: with the RPV-Saudi infection, almost 90% of the cells were virus antigen-positive, in comparison to the RPV-Egypt infection wherein only half of the culture was positive for virus glycoprotein; with RPV-RBOK, just over one-quarter of the cells showed evidence of virus antigen production by this time. It was also noted that cells infected with the Saudi isolate always stained slightly dimmer (lower values of mean fluorescent intensity) compared with the other two isolates. These observations were confirmed by immunofluorescence microscopy (data not shown).

In addition, monocyte-derived macrophages were analysed for their susceptibility to infection by these three RPV isolates. Similar results to those observed with infected monocytes were obtained (Fig. 2): the rate at which the highly virulent RPV-Saudi isolate spread through the culture with time p.i. was greater than when the attenuated RPV-RBOK vaccine strain

![Flow cytometric analysis of RPV-H glycoprotein expression on the surface of infected bovine monocytes. Cell were infected with three different isolates of RPV (RPV-RBOK, RPV-Egypt and RPV-Saudi) at an m.o.i. of 0-1 TCID50 per cell and stained with the anti-H glycoprotein MAb C1 at the times p.i. indicated. The numbers in each plot represent the number of positive cells stained by the MAb.](image)
was employed, with the mild RPV-Egypt isolate giving intermediate results. The replication of the viruses in the macrophages, regardless of the isolate used, appeared to progress at a lower rate than that in the monocytes. This was most evident at 6 days p.i. when, in the example shown, 56% of cells in the infected monocyte-derived macrophage cultures were virus antigen-positive, compared with 86% in the infected monocyte cultures.

These results were not due to a relative resistance of monocyte or macrophage subpopulations to infection by a particular RPV isolate. Continued incubation of the infected cultures revealed that > 95% of cells would contain detectable virus antigen by between day 7 p.i. (RPV-Saudi) and day 14 p.i. (RPV-RBOK; Rey Nores et al., 1995).

Influence of RPV infection of and replication in monocytes/macrophages on cell morphology

Similar results to the above were obtained when the staining was for the N protein of the virus, and analysis by immunofluorescence microscopy. Interesting observations on the morphology of the infected cells and the pattern of N protein expression were also forthcoming. Fig. 3 shows the results obtained at 10 days p.i. with infected monocyte-derived macrophages; similar observations were also made with the infected monocyte cultures. By this late time-point, all cells in the RPV-Saudi (Fig. 3c) and > 90% in the RPV-Egypt (Fig. 3b) cultures were staining for virus N protein. In contrast, < 20% of cells in cultures infected with the vaccine strain RPV-RBOK (Fig. 3a) were expressing nucleoprotein; no staining was found in the mock-infected controls (data not shown).

Numerous syncytia, in which the virus N protein was distributed throughout the cytoplasm, were present in all cultures. However, the size and complexity of syncytia differed with each strain. RPV-Saudi produced the largest syncytia (Fig. 3c), while the vaccine strain RPV-RBOK produced the smallest (Fig. 3a). Another feature of the infected monocytes and monocyte-derived macrophages was the formation of cell processes, with the infected cells acquiring a ‘stellate’ appearance (Fig. 3d, e). These cell processes or projections appeared to make contact with processes from other cells situated quite distant from each other in the culture. All strains could induce such processes, but their extrusion by an infected cell was more dominant in the infections involving RPV-Egypt.
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(Fig. 3b) and RPV-Saudi (Fig. 3d). As these infections progressed, the size of the syncytia increased, and the cell processes were lost, as can be seen in Fig. 3(c) which is a stage later in the infection than that shown in Fig. 3(d). In contrast, the RPV-RBOK infections, even late after infection, did not produce the same size or number of large syncytia seen with the other two isolates, but did retain the cell processes characteristic of infection by RPV (Fig. 3e). Without exception, the cell processes were intensively stained for virus N protein (Fig. 3).

To further analyse the morphological changes produced by the virus infection, replicate cultures at 10 days p.i. were fixed and stained with Diff-Quick. In mock-infected cultures, individual cells retained their characteristic appearance as macrophages (data not shown). Following infection with the vaccine strain RPV-RBOK, small syncytia of no more than three or four nuclei could be seen, but in general many cells in the culture resembled the macrophages in the mock-infected cultures (data not shown). The changes in cell morphology within the RPV-Egypt- and RPV-Saudi-infected cultures were more obvious, RPV-Egypt producing intermediate-sized syncytia comprising up to 10 nuclei, whereas RPV-Saudi produced syncytia that could contain up to 40 nuclei (data not shown).

Fig. 3. Immunofluorescent micrographs of RPV infection in monocyte-derived macrophages, demonstrating the virus-induced morphological effects, relative to the virus N protein-containing cells. (a) RPV-RBOK at 10 days p.i.; (b) RPV-Egypt at 10 days p.i.; (c) RPV-Saudi at 10 days p.i.; (d) RPV-Saudi at 5 days p.i., to show stellate cell formation; (e) RPV-RBOK at 14 days p.i., to demonstrate that stellate cell formation also occurs with this virus, but not the large syncytia typically found late after infection by RPV-Saudi or RPV-Egypt. The m.o.i. was 0.1 TCID<sub>50</sub> per cell. Fixation/permeabilization was at 10 or 5 days p.i., as mentioned above; staining for RPV-N protein used MAb IVB2-4, FITC–rabbit F(ab′)2 anti-mouse immunoglobulins and propidium iodide counterstain. Magnification 200×.
Production of infectious progeny virus in bovine monocytes and monocyte-derived macrophages

Monocytes and monocyte-derived macrophages infected with the three RPV isolates were compared for the amount of infectious progeny virus produced. Infectious virus was often first detected at 3 days p.i. in both infected monocytes and infected monocyte-derived macrophages. A low amount of virus was occasionally detected at 1 day p.i.; this was probably residual infectious material from the inoculum, particularly since it was primarily in the ECV fraction. Fig. 4 shows that there was a time-dependent (p.i.) increase in virus infectivity within both the extracellular and the cell-associated fractions. This was noted with all three virus strains, and in both infected monocyte and infected macrophage cultures. The highest virus titres were obtained with the RPV-Saudi isolate at 10 days p.i. in infected monocytes. The mild Egypt isolate produced lower titres than either RPV-Saudi or RPV-RBOK. Virus production in infected monocyte-derived macrophages was similar to that in infected monocytes but the appearance of infectious progeny was delayed, and the actual titres were most often higher with the monocyte cultures compared with the macrophages.

Effect of RPV infection on MHC class I, MHC class II and LFA-1 expression

Analyses of MHC class I, MHC class II and LFA-1 expression were performed during the initial 6 days following infection. Attempts to analyse these markers after day 6 p.i. were rendered problematic, due to the syncytia produced being too fragile to resist the preparative procedure necessary for the measurement of the molecules. The results obtained demonstrated that no significant changes were detectable in either MHC class I or LFA-1 expression, when control uninfected cultures were compared with cells infected by any of the three RPV isolates (Fig. 5: ‘BoLA I’ and ‘HLA I’; MHC class I expression identified by PT85A – a, e, i – and W6/32 – b, f, j – respectively; data not shown for LFA-1).

In contrast to MHC class I, a down-regulation of MHC
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Fig. 5. Flow cytometric analysis of BoLA (MHC) class I and II expression on the surface of infected bovine monocytes. Cells were mock infected (unfilled histogram), or infected (filled histogram) with RPV-RBOK (a–d), RPV-Egypt (e–h) or RPV-Saudi (i–l) at an m.o.i. of 0.1 TCID<sub>50</sub> per cell. Unfixed cells were stained at 6 days p.i. with MAb PT85A (anti-BoLA class I; a, e, i), W6/32 (anti-human HLA I, cross-reactive with BoLA MHC class I; b, f, j), H42A (anti-BoLA class II; c, g, k) and TH16B (anti-BoLA class II; d, h, l). All graphs were prepared as overlapping histograms of uninfected cells (unfilled histograms) over infected cells (filled histograms).

class II expression was observed in all infected cultures (Fig. 5: BoLA II; H42A – c, g, k – and TH16B – d, h, l). Although the MHC class II expression was lower than that for MHC class I on uninfected cells, the expression was clearly higher than the MOPC control antibody labelling (a fluorescence intensity of around 10 for the MHC class II labelling, compared to 1 for the MOPC control antibody). Comparable results were obtained whether MAb H42A or TH16B was used. The down-regulation was stronger with RPV-Egypt and RPV-Saudi (Fig. 5 g, h compared with Fig. 5 k, l); the peak shift obtained with the vaccine strain RBOK can only be described as slight (Fig. 5 c, d).

This down-regulation was not observed before 6 days p.i., suggesting that the effect becomes apparent only after a minimum number of cells had been infected by the virus. Comparison with Fig. 1 shows how down-regulation of MHC class II expression at 6 days p.i. relates to the number of infected cells in the cultures.

Discussion

Although different RPV isolates are grouped antigenically in a single serotype, they can show a variation in their virulence for the same host species, in terms of clinical symptoms and mortality. Experimental infections in vivo demonstrated that highly virulent strains, in comparison to so-called mild strains, replicated more rapidly, disseminated more efficiently, and produced more substantial alterations in infected tissues and organs (Taylor & Plowright, 1965; Wohlsein et al., 1993, 1995; Brown & Torres, 1994). The viraemic phase of the infection also differs between virulent
and avirulent strains of RPV (Plowright & Ferris, 1962a; Liess & Plowright, 1964), the latter producing lower-grade viraemia. Furthermore, due to the demonstration that the infective character of blood from RPV-infected animals was associated with the leukocytes (Plowright & Ferris, 1962a), it was speculated that lymphoid cells may vary in their susceptibility to RPV infection. Recent work has demonstrated that it is the monocytes and macrophages which are the main leukocyte target for in vitro RPV infection (Rey Nores et al., 1995). Lymphocytes can only be infected under particular circumstances such as following mitogen stimulation or interaction with endothelial cells (Rey Nores & McCullough, 1996). Differentiation of the monocytes into macrophages was not a pre-requisite for successful RPV infection, but it was noted that the infection progressed more readily in the monocyte cultures than in already differentiated macrophages.

The present work demonstrated that the highly virulent RPV-Saudi, the less virulent or ‘mild’ isolate RPV-Egypt and the attenuated avirulent vaccine strain RPV-RBOK could all infect mononuclear cells. Comparison of the infections showed that the kinetics of replication for each isolate was different: in terms of virus antigen production, the highly virulent RPV-Saudi isolate was the most efficient, followed by the mild RPV-Egypt and then the attenuated RPV-RBOK strain, which was the least efficient. A similar order appeared with respect to syncytia production, known to relate to intercellular spread of the virus. In contrast, with progeny virus production, which would be indicative of virus maturation, RPV-Egypt was the least efficient, with RPV-Saudi again being the most efficient.

Regardless of the virus strain, the production of both virus antigen and infectious virus progeny at the earlier time points p.i. was lower with infected macrophages compared with infected monocytes. Between days 6 and 10 p.i., the virus titres for infected macrophages approached those for infected monocytes. It was noted that this was not quite identical to the percentage of cells producing detectable antigen at 6 days p.i., being still lower with infected macrophages compared with infected monocytes. It was not until 10–14 days p.i., dependent on the RPV strain, that infected monocytes and macrophages had similar percentages of cells positive for virus antigen, not surprisingly since this was >90% in both cases. These results would suggest that replication in monocytes was initially the more efficient, regardless of the RPV strain. At later times in the infectious cycle, it was the infection in macrophages which became more efficient, due to the titres of infectious progeny produced, in relation to antigen production.

Such results were obtained whether monocytes or monocyte-derived macrophages were employed, although replication in the macrophages appeared to progress at a slower rate compared to that in the monocytes. Consequently, the characteristics of the infection were not due to a requirement for differentiation of monocytes into macrophages, nor to one particular isolate being more capable of interacting with a particular subpopulation of mononuclear cells. The reason for the more efficient infection of monocytes compared to macrophages may relate to the different levels of cytoplasmic, particularly lysosomal, activity in the two cell types, or to differences in endocytic and phagocytic activities. Nevertheless, these differences did not prevent the virus from eventually spreading to all cells in the culture.

Concerning the morphological changes induced in infected cells, again the virulent RPV-Saudi was more effective than the mild RPV-Egypt, which was in turn more effective than the avirulent RPV-RBOK. Interestingly, this was notable only for syncytia formation. When the formation of the processes, which tended to be packed with nucleocapsid protein, was studied, it was the mild RPV-Egypt which was seen to be more efficient at inducing stellate cells; the avirulent RPV-RBOK was still the least efficient. Such RPV strain-related differences in infected cell morphology, progeny virus titre and rate of progeny virus production were found only in monocytes and macrophages, but not in infected epithelial cell cultures (data not shown).

Consequently, the in vitro differences in virulence between the virulent RPV-Saudi and avirulent RPV-RBOK can be related to their rate of infection, efficiency at spreading intercellularly and capacity to induce morphological changes in infected cells. Although the mild RPV-Egypt strain can efficiently produce antigen and spread from cell to cell, it seems to be rather inefficient at maturation. Curiously, an amino acid change in the normally highly conserved cytoplasmic tail of the fusion protein responsible for fusion between the virus envelope and the host cell membrane, as well as between cells leading to syncytia formation, has been observed in RPV-Egypt (Evans et al., 1994).

RPV infection of monocytes was also seen to modulate MHC class II expression. A consistent down-regulation of MHC class II expression was found in all infected cultures, whereas no concomitant effect on class I or LFA-1 expression could be detected. By the time at least 50% of cells in the infected cultures were positive for virus antigen expression, the MHC class II down-regulation resulted in the cells of the culture appearing negative for this marker. During down-regulation, the cells moved as a homogeneous population; it was not that some cells down-regulated while others remained as positive as uninfected cells. Thus, both infected and uninfected cells in these cultures were being induced to down-regulate MHC class II. Such observations would imply a role for soluble factors produced by the infected cells. A number of other viruses can induce MHC class II down-regulation through interference with IFN-γ control processes (reviewed by Rinaldo, 1994).

These results on MHC class II down-regulation with RPV are in contrast to the results with another morbillivirus—MV—which increases MHC class II expression on infected human monocytes (Leopardi et al., 1993). How this relates to the down-regulation on RPV-infected monocytes remains to be determined. Why the two morbillviruses should have
diametrically opposed effects on MHC class II is not clear, although it may relate to a differential influence on the IFN-γ-dependent control mechanisms within the cells, as has been reported with cytomegalovirus modulation of MHC class II expression (Rinaldo, 1994).

Regardless of the mechanism, RPV-induced down-regulation of MHC class II expression is likely to interfere with efficient antigen presentation by the monocytes. It was interesting that the mild RPV-Egypt induced a greater degree of this down-regulation than the other two isolates. Due to the observation that RPV-Egypt appears to be less efficient at producing infectious virus compared with viral antigen, possibly relating to a slower or less efficient maturation compared with the other two isolates, a stronger down-regulation of MHC class II would be of benefit to the virus in its attempt to interfere with the host immune response. It might also aid the virus in establishing persistent infections, but to date there is no clear evidence that RPV can establish persistent infections.

In summary, bovine monocytes and monocyte-derived macrophages are susceptible to infection by isolates of RPV with different in vivo virulence. There is a relationship between the in vivo virulence of the isolate, including induction of clinical symptoms and rates of mortality, and its characteristics at producing viral antigen, inducing morphological changes and disseminating between the cells. These observations relate to the potential role which monocytes and monocyte-derived macrophages could play in RPV dissemination during natural infection.

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