Flow cytometric analysis of in vitro bluetongue virus infection of bovine blood mononuclear cells

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Cultures of adherent and non-adherent bovine peripheral blood mononuclear (PBM) cells were inoculated with bluetongue virus (BTV) serotype 10. Some cultures of non-adherent cells were stimulated with interleukin 2 (IL-2) and concanavalin A for 24 h prior to virus inoculation. Cells were harvested at various intervals up to 72 h after inoculation. A panel of leukocyte differentiation antigen-specific monoclonal antibodies (MAbs), specific for bovine CD2, CD4 or CD8, monocytes and granulocytes, B cells, γδ T cells or the IL-2 receptor (IL-2r), was directly conjugated to fluorescein isothiocyanate, and a MAb specific for the BTV major core protein VP7 was directly conjugated to phycoerythrin. Cells were labelled with conjugated MAbs in single- and double-label immunofluorescence studies to identify specifically the BTV-infected cells in inoculated cultures. The viability of cells was determined by propidium iodide exclusion, and all analyses were done using flow cytometry. Productive infection of cultures of PBM cells was confirmed by virus titration. The data revealed a clear difference between subsets of bovine PBM cells in susceptibility to infection with BTV in vitro. Monocytes were readily infected with BTV, as were stimulated CD4+ cells, and infection was cytopathic to monocytes and stimulated lymphocytes. The proportion of infected cells decreased after 24 h and virus titres dropped markedly by 72 h in all cultures. CD4+ cells in cultures of unstimulated non-adherent cells inoculated with BTV showed increased expression of IL-2r. The possible relevance of these findings to the pathogenesis of BTV infection of cattle is discussed.

Introduction

Bluetongue virus (BTV) is the causative agent of bluetongue, an insect-transmitted disease of ruminants (Spruell, 1905; Moulton, 1961; Bowne, 1971; Erasmus, 1975; Hourrigan & Klingsporn, 1975). BTV infection of cattle typically is subclinical and is characterized by prolonged viraemia (Du Toit, 1962; Luedke et al., 1969; MacLachlan & Fuller, 1986; MacLachlan et al., 1987; Richards et al., 1988), during which the virus invariably is cell-associated (Luedke, 1970; Colisson & Barber, 1983; Morrill & McConnell, 1985; Parsonson et al., 1987). Recent studies have established that mononuclear inflammatory cells might be important to the pathogenesis of bluetongue in cattle. Using a variety of in vitro techniques, Whetter et al. (1989) have identified BTV-infected cells in cultures of adherent bovine peripheral blood mononuclear (PBM) cells, and virus has been transiently isolated from PBM cells during experimental infection of calves (Whetter et al., 1989; MacLachlan et al., 1990). Virus is present in high titre in lymph nodes soon after inoculation of calves (MacLachlan et al., 1990), indicating that lymphoid tissue is an important site for initial virus replication. BTV infection of long-term T lymphocyte cultures has been demonstrated. It has been suggested that virus replication in bovine T cells is limited to those cells undergoing blastogenesis, but the specific subset of cells infected has not been established (Stott et al., 1990). The precise role of mononuclear inflammatory cells in the pathogenesis of BTV infection of cattle remains to be adequately described.

The purpose of this study was to characterize further the interaction between BTV and bovine PBM cells in vitro. Dual colour flow cytometric analysis of BTV-infected cells was done with monoclonal antibodies (MAbs) specific for BTV and bovine PBM cell subpopulations. The specific objectives were to identify definitively the infected cells in cultures of freshly isolated PBM cells inoculated with BTV, including short-term cultures undergoing blastogenesis, and to examine the effect of BTV infection on monocytes and lymphocyte subsets in these cultures. Monocyte- and B cell–virus interactions were evaluated in adherent PBM cell
cultures, and the effects of BTV on resting and blastic T cells were evaluated in unstimulated and stimulated non-adherent PBM cell cultures, respectively.

Methods

**Viruses.** Highly plaque-purified BTV-10 was used in this study. The passage history of this virus has been described previously (MacLachlan et al., 1987). Stock cultures of the virus were propagated in BHK-21 cells.

**BTV-specific MAbs.** The production and characterization of a MAb to BTV structural protein VP7 have been described previously (Wheater et al., 1989). MAb to VP7 was directly conjugated to R-phycocerythrin (PE) as described (Oi et al., 1982).

**Bovine leukocyte differentiation antigen-specific MAbs.** The following MAbs were used: IL-A42, specific for bovine CD2+ cells (Teale et al., 1987); IL-A11, specific for bovine CD4+ cells (Baldwin et al., 1996); IL-A51, specific for bovine CD8+ cells (Ellis et al., 1986); IL-A24, specific for bovine monocytes and granulocytes (Ellis et al., 1988); IL-A29, specific for bovine CD4+, CD8+, γδ T cell receptor+ cells (γδ T cells, Clevers et al., 1990). These MAbs were produced at the International Laboratories for Research in Animal Diseases and obtained through the ATCC, with the exception of IL-A24 (generously supplied by Dr J. A. Ellis, Wyoming State Veterinary Laboratory, Laramie, Wyo., U.S.A.). Also used were CC21, specific for bovine B cells (Naessens et al., 1990; generously supplied by Dr C. J. Howard, Institute for Animal Health, Compton Laboratory, Compton, Newbury, U.K.) and UC-2C2, specific for the bovine low affinity interleukin 2 receptor (IL-2R) expressed on stimulated T cells (Taylor et al., 1992). All leukocyte-specific MAbs were directly conjugated to fluorescein isothiocyanate (FITC) as described (Goding, 1986). MAbs Ca13.1E4 and Ca9.AG5 (generously supplied by Dr P. F. Moore, Davis, Ca., U.S.A.), specific for canine CD4+ and CD8a, respectively, and not reactive with bovine leukocytes, were conjugated to FITC and PE and used as irrelevant control antibodies.

**Preparation and infection of bovine PBM cells.** Venous blood was collected in acid citrate dextrose solution from a colostrum-deprived calf reared in insect-security isolation facilities. PBM cells were separated from theuffy coat fraction by centrifugation through ficoll and sodium diatrizoate (Histopaque; Sigma) at 900 g for 30 min. Cells were cultured in Dubbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% foetal bovine serum (FBS) and antibiotics in plastic flasks for 4 h after which non-adherent cells were removed by washing. Adherent cells were cultured at a density of 2 x 10^5 to 4 x 10^6 cells/cm^2 in the same medium. Non-adherent cells were maintained in plastic flasks at a density of 2 x 10^5 to 4 x 10^6 cells/ml in DMEM supplemented with 20% FBS and non-essential amino acids, glutamine and sodium pyruvate, with or without recombinant human IL-2 (100 units/ml; generously supplied by Cetus Corporation) and concanavalin A (ConA, 1 μg/ml; Sigma). Preliminary studies revealed that ConA induced more blastogenesis in cultures of non-adherent PBM cells than did either phytohaemagglutinin or pokeweed mitogen. Stimulated non-adherent cultures were exposed to 0.02 TCD50/cell or mock-infected with DMEM. Preliminary data revealed that a m.o.i. greater than 0.02 did not significantly increase the percentage of BTV-infected cells in inoculated cultures of bovine PBM cells. Virus was allowed to adsorb for 1 h after which cells were washed and cultured in DMEM supplemented as described.

**Immunofluorescence and flow cytometry.** Adherent cells were removed from flasks by incubation in 10 mM-EDTA for 5 to 10 min at 4°C. Cells were washed in PBS containing 0.01% sodium azide (buffer) and preincubated with 10% normal mouse serum to reduce non-specific binding of MAbs. All antibody incubations were performed for 30 min in the dark at 4°C. Samples stained only for cell identification were incubated with one of the panel of FITC-conjugated leukocyte differentiation antigen-specific MAbs or a control MAb, then washed in buffer. These cells were then incubated with 50 μg/ml propidium iodide for at least 1 h prior to flow cytometric analysis. Cells stained for BTV antigens first were fixed and permeabilized with 90% methanol for 30 min, followed by 0.5% non-ionic detergent (Triton X-100; Mallinckrodt) for 3 min. Cells were washed twice in buffer and incubated with PE-conjugated control MAb or MAb to VP7. Cells to be double-labelled were first incubated with one of the panel of FITC-conjugated leukocyte differentiation antigen-specific MAbs, then fixed and permeabilized, and finally incubated with PE-conjugated MAb to VP7. To confirm the specificity of staining with BTV-specific MAb, adherent PBM cells were infected with bovine herpesvirus type 1 (BHV-1). Infection with BHV-1 was confirmed by indirect immunofluorescence with polyclonal bovine antisera (generously supplied by Dr A. Castro, California Veterinary Diagnostic Laboratory Systems, Davis, Ca., U.S.A.). Double-label immunofluorescence staining also was done on BTV-infected cultures of PBM cells isolated from two additional colostrum-deprived calves aged 8 weeks.

Cultures of adherent, and of unstimulated and stimulated non-adherent bovine PBM cells were inoculated with BTV-10, or mock-infected, and harvested at 1, 16 and 36 h after inoculation. The proportions and viability of PBM cell subpopulations present in these cultures were determined by labelling cells with each FITC-conjugated leukocyte differentiation antigen-specific MAb and propidium iodide, as described. Viable cells were identified by exclusion of propidium iodide as determined by flow cytometry (Krishan, 1975). The susceptibility of cells in adherent and unstimulated and stimulated non-adherent PBM cell cultures to BTV infection was also evaluated. Cultures were harvested at 1, 4, 8, 12, 16, 20, 24, 30, 36 and 48 h after inoculation, and cells were fixed and permeabilized, and stored at 4°C. Preliminary data indicated no decrease in intensity of fluorescence of fixed samples stored prior to staining. Stained samples were stained with PE-conjugated control MAb or MAb to VP7 and analysed by flow cytometry to determine the percentage of BTV-infected cells present at each time. Double-label immunofluorescence staining was performed on PBM cells harvested at 1 and 16 h after virus inoculation to establish the phenotype of BTV-infected cells. Inoculated cells were harvested, labelled with each FITC-conjugated cell-specific MAb and PE-conjugated MAb to VP7 as described, and analysed by flow cytometry.

All immunofluorescence analyses were performed on a FACScan flow cytometer (Becton Dickinson) using 15 mW laser emitting at 488 nm. In general, 10000 cells/sample were analysed. Data were acquired and analysed with the Lysys II computer program (Becton Dickinson) using logarithmic amplification for fluorescence intensity. Cellular debris was excluded from analysis by raising the threshold for forward scatter detection. Data were analysed either as histograms depicting fluorescence intensity of a single colour against cell number, or as dual colour smoothed contour plots depicting fluorescence intensity due to FITC against that due to PE. Background fluorescence was determined by raising the threshold for forward and side scatter and analysed with the Lysys II computer program (Becton Dickinson) using a 15 mW laser emitting at 488 nm.
Results

Adherent PBM cells

Numerous cells in adherent cultures developed pseudopodia and foamy cytoplasm characteristic of macrophages. Immunofluorescence labelling with cell-specific MAb and propidium iodide indicated that cells in this population were 90% viable at the time of virus inoculation, and were primarily monocytes (42%) and B cells (30%). The proportion of viable cells in the virus-inoculated population at 16 h (82%) was less than that in mock-infected cultures (90%). This decrease was attributed to death of monocytes in BTV-infected populations, as only 33% viable monocytes were present in the infected population as compared to 59% in the control culture (Fig. 1). The difference in the proportion of viable monocytes in these two populations was less profound at 36 h (39% and 50%, respectively), and was accompanied by a relative increase in the proportion of viable cells in infected populations. Minimal c.p.e. was evident in infected adherent cultures at 24 h and was manifest principally as the rounding up and loss of adherence of scattered individual adherent cells. Immunofluorescence labelling of cells harvested at intervals after virus inoculation using MAb to VP7 peaked at 10% of the population at 24 h and then gradually decreased to 5% at 48 h after inoculation (Fig. 2b). The virus titre of cells from inoculated adherent cultures increased more than 30-fold by 36 h after inoculation and then declined markedly (Fig. 2a). The virus titre of cells from inoculated adherent cultures included more than 30-fold by 36 h after inoculation and then declined markedly (Fig. 2b). Of all cells from adherent cultures, 7% were double-labelled with MAb IL-A24 (monocyte-specific) and MAb to VP7 at 16 h; 15% of monocytes had strong reactivity with MAb to VP7 as compared to only 2% after virus adsorption (Fig. 3a, b). Essentially all cells reacting with MAb to VP7 reacted with MAb IL-A24 (upper right quadrant of contour plot), reflecting the lack of infection of other cell types, principally B cells, in adherent cultures. Similarly, no double-labelled cells were detected in samples stained with CC21 (B cell-specific) and MAb to VP7 (data not shown). Double-label immunofluorescence staining of cells from BTV-inoculated adherent cultures of PBM cells isolated from the two additional calves gave results comparable to those obtained with cells from the principal calf, although the actual percentage of infected monocytes varied (data not shown). Staining of BTV-infected cells with MAb to VP7 was specific; adherent cells infected with BHV-1 had c.p.e. and decreased viability similar to that of BTV-infected cells, but did not react with MAb to VP7, and BTV-infected cells did not react with control MAb.

Unstimulated non-adherent PBM cells

Unstimulated non-adherent cells appeared to be quiescent in culture and did not exhibit obvious c.p.e. after inoculation with virus. Immunofluorescence labelling with cell-specific MAb and propidium iodide indicated that these cultures included 72% viable T cells at the time of virus inoculation, with 17% being γδ T cells. There was no clear difference in the proportion of individual mononuclear cell subsets or viability in virus-inoculated cultures as compared to control populations at 16 and 36 h after inoculation (Table 1). However, the proportion of IL-2r+ (UC-2C2+) cells at 16 h was 27% in virus-inoculated cultures, as compared to 15% in mock-infected cultures (Fig. 4). A small percentage of cells in unstimulated non-adherent cultures reacted with MAb to VP7. Infection peaked at 3% of the population at 24 h...
Fig. 2. Percentage of infected cells (a) and titres of BTV (b) in adherent (▲), unstimulated non-adherent (■), and IL-2- and ConA-stimulated non-adherent (●) cultures of bovine PBM cells at various times after inoculation with BTV. The percentage of infected cells was determined by reactivity with PE-conjugated MAb to VP7 after fixation with methanol and non-ionic detergent. Virus titres were determined by microtitration of sonicated cells and supernatant on monolayers of BHK-21 cells.

after inoculation, and decreased to only 1% at 48 h (Fig. 2a). An analysis gate was used when studying these cells by flow cytometry, to eliminate the possible effect of contaminating monocytes; this gate was sufficiently broad that there was negligible exclusion of lymphocytes. The virus titre of cells from unstimulated non-adherent cultures increased almost 20-fold by 24 h after inoculation and then decreased (Fig. 2b). Double-label immuno-fluorescence analysis at 16 h identified BTV-infected cells as being principally of the CD4+ phenotype (Fig. 3d) and these were largely IL-2r+. Few BTV-infected CD4+ cells were identified (upper left quadrant of contour plot), and negligible numbers of CD8+ or γδ T cells reacted with MAb to VP7 (data not shown). Similar results were obtained by double-label immuno-fluorescence analysis at 16 h of unstimulated (c, d) and IL-2- and ConA-stimulated (e, f, g, h) cultures of bovine PBM cells at 1 (a, c, e, g) and 16 (b, d, f, h) h after virus inoculation. Cells in upper right quadrants represent double-labelled cells. Numbers represent the percentage of cells in each quadrant. Note the lack of cells in the upper left quadrants of (a), (b), (c) and (d), reflecting negligible infection of B cells (a, b), and CD8+ and γδ T cells (c, d). Note also the small percentage of moderately fluorescent cells in the upper left quadrant of (f), reflecting infection of CD4+ cells (CD8+ and γδ T cells) in stimulated cultures.
BTV infection of bovine mononuclear cells

Fig. 4. Histograms depicting fluorescence plotted against cell number, representing the proportion of cells in unstimulated cultures of non-adherent bovine PBM cells reacting with FITC-conjugated UC-2C2 (IL-2r) at 16 h after virus inoculation. Cultures were mock-infected (a) or inoculated with BTV (b).

Table 1. Proportions of subsets of viable T cells in unstimulated, and IL-2- and ConA-stimulated cultures of non-adherent bovine PBM cells

<table>
<thead>
<tr>
<th>Culture conditions*</th>
<th>Unstimulated</th>
<th></th>
<th></th>
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<th>IL-2 + ConA stimulated</th>
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<td>16 h</td>
<td>36 h</td>
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<td>27</td>
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<td>IL-2r</td>
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<td>27</td>
<td>15</td>
<td>23</td>
<td>12</td>
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* Cultures were inoculated with BTV (BTV) or medium (CN) in vitro. All values are percentages determined by immunofluorescence labelling with cell-specific MAb. Viability was determined by propidium iodide exclusion and data were analysed by flow cytometry.
cells, as shown in the upper left quadrant in Fig. 3 (e, f). Only 4% of cells labelled with MAb IL-A51 (CD8-specific) and MAb to VP7 were double-labelled at 16 h, as were 3% of all cells stained with MAb IL-A29 (CD8 T cell-specific) and MAb to VP7 (data not shown). Similar results were obtained with cells from BTV-inoculated stimulated non-adherent PBM cell cultures from the two additional calves, although the levels of infected lymphocytes of each subset varied, as determined by double-label immunofluorescence staining (data not shown).

Discussion

This study was undertaken to characterize BTV infection of bovine PBM cells in vitro. An extensive panel of MAbs directly conjugated to fluorochromes enabled the specific identification of BTV-infected cells using dual colour immunofluorescence labelling. Flow cytometry provided sensitive quantitative analysis of cells in the various PBM cell cultures. It was necessary to use highly plaque-purified virus to preclude infection with other viruses or mixed BTV strains, but it is appreciated that wild-type virus may not act in a fashion identical to this cell culture-adapted strain of BTV-10.

BTV productively infected cultures of bovine PBM cells, as determined by virus titration and immunofluorescence labelling. The virus titre increased 20- to 30-fold in each culture by 24 to 48 h compared to the titre at 1 h post-inoculation; the greatest increase in titre was in cells from adherent and stimulated non-adherent cultures. Immunofluorescence staining indicated that at least 10% of all cells from adherent and stimulated non-adherent cultures were infected by 24 h, although minimal infection was observed in cells from unstimulated non-adherent cultures. It was apparent that not all cells in cultures of bovine PBM cells were susceptible to BTV infection in vitro. The proportion of fluorescence-positive cells and virus titres of PBM cell cultures decreased markedly in all cultures by 48 and 72 h, respectively, after inoculation. Preliminary studies demonstrated that the percentage of infected cells was not significantly increased by increasing the m.o.i., as also observed by Whetter et al. (1989). BTV infection resulted in the death of susceptible cells, as determined by propidium iodide staining, which were subsequently excluded from analysis by flow cytometry. Presumably the lack of significant rounds of secondary virus replication resulted in the decrease in virus titre of PBM cell cultures clearly occurring over time.

The data clearly demonstrate a difference in susceptibility of subsets of bovine PBM cells to in vitro BTV infection. Bovine monocytes were readily infected with BTV, as previously reported (Whetter et al., 1989). Immunofluorescence labelling revealed that 15% of monocytes in adherent cultures were infected with BTV at 16 h after virus inoculation. CD4+ T cells were also susceptible to BTV infection, although less so without prior mitogenic stimulation, whereas CD8+ cells and γδ T cells were infected only if stimulated with IL-2 and ConA to induce blastogenesis prior to inoculation. The mitogen used did not result in B cell stimulation, and resting B cells were not infected with BTV.

This and earlier data suggest that monocytes and tissue macrophages are important in the pathogenesis of BTV infection of cattle. BTV is transmitted via the bites of infected insects (Bowne, 1971) and virus probably travels from the site of inoculation via afferent lymphatics to the draining lymph node, where initial virus replication occurs (MacLachlan et al., 1990). Veiled cells may contribute to the transport of virus in afferent lymphatics; these cells have some phenotypic and functional similarities to blood monocytes (McKeever et al., 1991). Lymph node macrophages could also be a site of virus replication as they constitute about 10% of all cells in lymph node suspensions (Ellis et al., 1987).

The susceptibility of CD4+ cells to in vitro BTV infection and the isolation of BTV in high titre from lymphoid organs of experimentally infected animals (MacLachlan et al., 1990) indicate that CD4+ cells may also play an active role in BTV infection of cattle, as suggested previously (Stott et al., 1990). CD4 is found on approximately 35% of bovine afferent lymph cells (Emery et al., 1987), 70% of thymocytes and 30% of PBM cells (Baldwin et al., 1986), and CD4+ cells are abundant in the paracortical region of lymph nodes (Baldwin et al., 1986). IL-2- and ConA-stimulated T cells were readily infected with BTV in vitro, and BTV infection of stimulated CD4+ cells in vivo might result in substantial amplification of virus titre. Veiled cells are absent from efferent (central) lymph (Hall & Morris, 1963; MacKay et al., 1988); infected CD4+ cells could facilitate the spread of BTV from afferent lymph and lymph nodes to peripheral blood via efferent lymphatics. Spread of virus to peripheral tissues presumably is facilitated by infected monocytes and CD4+ cells, as well as erythrocytes, in peripheral blood (Whetter et al., 1989; MacLachlan et al., 1990).

CD4+ cells in unstimulated non-adherent cultures inoculated with BTV showed increased expression of IL-2r, as compared to cells in control inoculated cultures. Activation of memory T cells specific for BTV was not responsible as the donor animal was colostrum-deprived and raised in isolation to prevent BTV infection. There was no obvious blastogenesis in virus-inoculated unstimulated cultures of PBM cells, and Stott et al. (1990) have observed that minimal blast formation occurs in stimulated non-adherent cultures of bovine PBM cells.
previously exposed to BTV. The significance of this increased expression of IL-2r on CD4+ cells is not known.

γδ T cells and CD8+ cells were minimally infected with BTV in vitro, as compared to monocytes and CD4+ cells, and the contribution of these cells to the pathogenesis of BTV infection of cattle is probably minor. However, γδ T cells are a prominent cell type in the ruminant immune system, especially in neonates (Hein & MacKay, 1991), whereas CD8+ cells represent approximately 20% of PBM cells and 70% of thymocytes, and are present in T cell-dependent regions of lymph nodes and spleen (Ellis et al., 1986). Infection of γδ or CD8+ T cells obviously could contribute to the dissemination of BTV through the body of infected cattle.

This study has demonstrated a differential sensitivity of bovine PBM cell subsets to BTV infection in vitro. The methodology developed here is presently being adapted for in vivo studies to define precisely the roles of subsets of PBM cells in the pathogenesis of BTV infection of cattle.

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