Bluetongue Virus Infection of Bovine Monocytes

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SUMMARY

Cultures of adherent and non-adherent bovine blood mononuclear cells were infected with bluetongue virus (BTV) serotype 10. Production of BTV proteins in mononuclear cell cultures was detected by immune precipitation of viral proteins from [35S]methionine-labelled extracts of these cells, by immunofluorescence staining of cells using monoclonal antibodies (MAbs) to BTV proteins VP7 and NS2, and by flow cytometry with MAbs to VP2, VP7, NS1 and NS2. BTV-infected cells were most numerous in cultures of adherent mononuclear cells; infected cells were initially identified as monocytes on the basis of their morphology, and size and scatter characteristics as determined by analysis with a fluorescence-activated cell sorter (FACS). The majority of adherent mononuclear cells with these scatter characteristics were confirmed to be monocytes by FACS analysis with a MAb specific for bovine monocytes. Identification of BTV-infected adherent mononuclear cells as monocytes was further established by double immunofluorescent labelling, as infected adherent cells reacted with the MAb specific for bovine monocytes, and with another MAb specific for class II antigen. Infection of adherent mononuclear cells was also confirmed by transmission electron microscopy, as BTV virions and tubules were present in lysates of cultures of BTV-infected adherent mononuclear cells and within the cytoplasm of adherent cells. In contrast, BTV proteins were detected in few cells identified as lymphocytes on the basis of their scatter characteristics, and mean fluorescence of such cells was considerably less than that of BTV-infected monocytes. Viraemia persisted until 35 days after inoculation of a colostrum-deprived calf inoculated with BTV. Virus was isolated from blood mononuclear cells at 1 week after infection of the calf, but not thereafter. BTV infection of blood mononuclear cells was demonstrated until 9 days after inoculation by indirect immunofluorescence staining of mononuclear cells. In contrast, virus was consistently isolated from erythrocyte-enriched preparations throughout viraemia in titres comparable to those in whole blood. These results indicate that although bovine monocytes are readily infected in vitro with this strain of BTV serotype 10, infection of blood monocytes is unlikely to be responsible for the prolonged viraemia that consistently occurs in BTV-infected cattle.

INTRODUCTION

Bluetongue virus (BTV)-infected cattle usually have a prolonged viraemia, which persists for several weeks despite the concurrent presence of neutralizing antibody in serum (DuToit, 1962; Luedke et al., 1969; MacLachlan & Fuller, 1986; MacLachlan et al., 1987; Heidner et al., 1988; Richards et al., 1988). Association of BTV with blood cells might allow the virus to avoid neutralization and subsequent elimination (Luedke, 1970; MacLachlan et al., 1987). Although it is well established that BTV is strongly cell-associated during viraemia, the specific cell type with which BTV is preferentially associated is uncertain. BTV serotype 11 was isolated from both buffy coat cells and erythrocytes during viraemia in experimentally infected calves, but the
virus was most consistently isolated at the highest titres, from red blood cells (Luedke, 1970). In contrast, BTV was isolated from both the buffy coat and erythrocyte fractions of peripheral blood of cattle infected with BTV serotype 20, and was not preferentially associated with erythrocytes (Parsonson et al., 1987). Collisson & Barber (1983) isolated similar titres of BTV from all blood cell fractions, including erythrocytes, polymorphonuclear cells, and adherent and non-adherent mononuclear cells, obtained from the peripheral blood of cattle infected with BTV serotype 11. Morrill & McConnell (1985) examined by transmission electron microscopy blood cells from calves infected with a mixture of BTV serotypes 10, 11, 13 and 17, and identified virus-like particles in monocytes and, less often, lymphocytes.

The purpose of this study was to determine, using a variety of techniques, whether BTV serotype 10 can productively infect bovine blood mononuclear cells in vitro, and to determine whether BTV-infected mononuclear cells could be identified in the peripheral blood of a calf inoculated with the same strain of BTV.

METHODS

Virus and cells. Baby hamster kidney (BHK-21), African green monkey kidney (Vero) and P3-X63-Ag8-653 mouse myeloma (P3) cells were obtained from the American Type Culture Collection. The passage history of the strain of BTV serotype 10 used in this study has been previously described (MacLachlan et al., 1987). Stock cultures were propagated in BHK-21 cells. Purified virus was prepared by banding on a continuous sucrose density gradient (Huismans et al., 1987).

Bluetongue virus-specific monoclonal antibodies (MAbs). The production of BTV-specific MAbs, including the sensitization of mice used for production of hybridomas, has been described (Heidner et al., 1988). Hybridoma supernatants were screened by indirect immunofluorescence and neutralization assays. Neutralization assays were done as previously described (Heidner et al., 1988). Immunofluorescence staining was done on 80% acetone-fixed, BTV-infected monolayers of Vero cells grown on chamber slides (Miles Laboratories). Monolayers were incubated with undiluted hybridoma supernatants followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Kirkegaard & Perry). Hybridomas were grouped according to the staining pattern they produced in infected cells, and one or more hybridomas which represented each staining group was selected for cloning.

The protein specificity of each MAb was determined by immune precipitation of [35S]methionine-labelled, ultracentrifuged lysates of BTV-infected BHK-21 cells (Mitchell et al., 1980; Appleton & Letchworth, 1983; Heidner et al., 1988). Ultracentrifugation also removed BTV non-structural protein NS1, so that immune precipitation of the MAb specific for NS1 necessitated the use of labelled lysates which had not been ultracentrifuged. Proteins were resolved on a 10% polyacrylamide gel and were identified by comparison to [35S]methionine-labelled proteins in gradient-purified BTV, and those proteins precipitated from BTV-infected BHK-21 cell lysates by a hyperimmune rabbit antiserum to BTV serotype 10 (Heidner et al., 1988; Richards et al., 1988). The electrophoretic mobility of the immunoprecipitated proteins was compared to that of Mr markers to determine their size and identity (MacLachlan et al., 1987).

Preparation and infection of bovine blood mononuclear cells. Mononuclear cells were isolated from the peripheral blood of colostrum-deprived, isolation-reared calves that were not infected with BTV and which were seronegative for this virus. Mononuclear cells were separated from whole blood by centrifugation through Sepracell-MN medium (Sepratech Corporation), and cultured in RPMI supplemented with 10% foetal calf serum and antibiotics. Mononuclear cells were cultured in plastic flasks for 4 h at 37 °C to facilitate adherence, and non-adherent cells were removed by repeated washing. Non-adherent cells were cultured at a density of 1 × 10^6 cells/ml and adherent cells at 2 × 10^5 to 5 × 10^5 cells/cm^2.

Cultures of adherent and non-adherent bovine blood mononuclear cells were infected with BTV at an m.o.i. of 0.02 p.f.u./cell. Control mononuclear cell cultures were mock-infected with medium. Cultures were harvested immediately after virus adsorption, and at 24, 48, 72 and 96 h after infection. Cell and supernatant fractions from the different cultures were harvested separately, and virus titres in each were determined by microtitration on Vero cells essentially as previously described (MacLachlan et al., 1984).

For immunofluorescence staining of cultured bovine blood mononuclear cells, adherent cells were grown on chamber slides and non-adherent cells were grown in suspension, and then pelleted onto glass slides prior to fixation. All cultures were infected with BTV at an m.o.i. of 1-0 p.f.u./cell. Cells were fixed in 80% acetone at 24, 44 and 120 h post-infection (p.i.) and subjected to indirect immunofluorescence staining. Only the MAbs specific for VP7 and NS2 were used in these studies, as these two MAbs consistently gave the most obvious fluorescence in monolayers of BTV-infected Vero cells. BTV-infected Vero or P3 mouse myeloma cells were included as a positive control, and MAb OX-19, a rat thymocyte-specific MAb which does not react with bovine cells (courtesy of Dr P. Carter and Ms K. Beegle, North Carolina State University) was used as a negative MAb control. To minimize...
background fluorescence, the MAbs were incubated with bovine serum albumin before staining and the secondary antiserum was preincubated with calf serum which was free of BTV-specific antibodies. Cultures were examined on a microscope with the requisite filter for epifluorescence of FITC.

The BTV-infected adherent mononuclear cells were further characterized by double labelling for both BTV proteins and either class II antigen of the bovine major histocompatibility complex (MHC) or an antigenic domain present on bovine monocytes that express class II MHC antigen. Cultures of adherent blood mononuclear cells were fixed in 80% acetone at 15 to 18 h after inoculation with BTV or mock infection. In all double-labelling studies, BTV infection of cells was detected with FITC-conjugated MAb to VP7. Class II MHC antigen was identified with biotinylated MAb 2D16, a broadly species cross-reactive MHC class II antigen-specific MAb (Moore et al., 1986), and avidin-conjugated Texas Red fluorochrome (Vector Laboratories). The bovine class II monococyte-specific antigenic domain was identified by indirect immunofluorescence staining using MAbs DH59B (from VMRD), biotinylated rabbit anti-mouse IgG (Zymed Laboratories) and avidin-conjugated Texas Red. Monoclonal antibody DH59B also reacts with granulocytes, but neither T cells nor B cells, and is, therefore, not directed against class II antigen itself (Davis et al., 1987). Cultures were examined on a microscope using filters appropriate for epifluorescence of Texas Red and FITC.

Production of BTV proteins in infected cells was identified by immune precipitation. BHK-21 cells and cultures of similar numbers of adherent and non-adherent blood mononuclear cells were infected at an m.o.i. of 1·0 p.f.u./cell. At approximately 12 h p.i., 35S-labelled methionine was added. The cells were incubated for a further 1·5 h, rinsed and then lysed with NET/NP40 buffer. The lysates were centrifuged and radiolabelled proteins were resolved by polyacrylamide gel electrophoresis (MacLachlan et al., 1987). Viral proteins in these lysates were also identified by immune precipitation using the hyperimmune polyclonal rabbit antiserum (Heidner et al., 1988; Richards et al., 1988).

Flow cytometry. A Becton-Dickinson fluorescence-activated cell sorter (FACS 440) equipped with a Spectra-Physics 5 W argon laser was used for all FACS analysis. In each experiment the laser was tuned to 488 nm and operated at 400 mW output. A band pass 530/30 and a long pass filter were used for the detection of green and red fluorescence respectively. For each sample, at least 15 × 10^3 cells were measured and the data were stored in list mode fashion. Data analyses were performed with the Cellsoft program (Cellsoft Biotechnologies). Gates were established on forward angle scatter to exclude small cellular debris from the analyses of specific fluorescence. In all FACS analyses, the level of fluorescence exhibited by BTV-infected cells stained with the rat thymocyte-specific MAb OX-19 was considered to be background attributable to non-specific staining. MAb OX-19 consistently failed to react with BTV antigens and produced similar levels of fluorescence when used to stain either infected or uninfected cells. Cells exhibiting greater fluorescence than background were considered positive. The percentage of cells with fluorescence above the background level and the mean fluorescence of cells in each population were determined for each sample.

Mononuclear cell cultures were infected at an m.o.i. of 0·5 p.f.u./cell. This m.o.i. was selected as it produced minimal background fluorescence attributable to binding of viral proteins in the challenge inoculum. Cells in the cultures were fixed with 3·7% formaldehyde at 18 h p.i. and stained by indirect immunofluorescence using the MAbs specific for VP2, VP7, NS1, NS2 and rat thymocytes (OX-19). The fixative and optimal time of fixation were selected on the basis of results of preliminary studies using BTV-infected P3 cells. Size and granularity of mononuclear cells were assessed by forward and side (90°) angle scatter.

Cultured mononuclear cells, both adherent and non-adherent, were further characterized by FACS analysis after indirect immunofluorescent staining of these cells with MAb DH59B, specific for bovine monocytes that express class II MHC antigen. Mononuclear cells that reacted with MAb DH59B were stained with FITC-conjugated goat anti-mouse IgG (Kirkegaard & Perry).

Electron microscopy. Adherent and non-adherent cultures of bovine mononuclear cells were infected at an m.o.i. of 1·0 p.f.u./cell. The cells were collected at 12 and 18 h p.i., pelleted and fixed in Trump's fixative before processing for transmission electron microscopic examination.

BTV infection of calves. Two colostrum-deprived calves were reared in insect-secure isolation facilities. Calves were free of BTV infection, and seronegative to this virus at the time of inoculation. One calf was intravenously inoculated with 4·6 × 10^4 p.f.u. of fluorocarbon and ether-extracted BTV (MacLachlan et al., 1984). The other calf was inoculated with ether and fluorocarbon-extracted uninfected BHK-21 cells. Blood was collected at weekly intervals from the calves, and the titre of virus in whole blood samples was determined by isolation in BHK-21 cells, as previously described (MacLachlan et al., 1987). In addition, peripheral blood cells were separated by centrifugation through Seprackel-MN medium. Cells in the mononuclear and erythrocyte fractions were counted, lysed by resuspension in hypo-osmolar (0·002 M) Tris–HCl buffer pH 8·8, and titres of virus in each cell fraction were determined by titration in BHK-21 cells. Aliquots of the different blood cell fractions were pelleted onto glass slides and subjected to indirect immunofluorescence staining using MAbs to VP7 and NS2. Immunofluorescence staining of enriched blood cell fractions was done at weekly intervals after infection, and at 9 days after inoculation.
RESULTS

**BTV-specific MAbs**

Four MAbs which gave distinct immunofluorescence staining characteristics in BTV-infected Vero cells were selected and further characterized. The MAbs were specific for VP2, VP7, NS1 and NS2, as determined by immune precipitation from lysates of BTV-infected BHK-21 cells (Fig. 1). The MAb to VP2 also neutralized infectivity of BTV. Specificity of the MAbs to BTV was confirmed by preincubating the MAbs with gradient-purified BTV or lysates of BTV-infected BHK-21 cells before indirect immunofluorescence staining of fixed monolayers of BTV-infected Vero cells. Staining with MAbs to all four BTV proteins was blocked by preincubating the MAbs with infected BHK-21 cell lysate, whereas preincubation with purified BTV blocked only the staining of MAbs specific for VP2 and VP7. Preincubation of the MAbs with uninfected BHK-21 cell lysate did not affect their staining of BTV-infected cells. The MAbs to VP7 and NS2 consistently gave the most obvious fluorescence after indirect immunofluorescence staining of acetone-fixed BTV-infected Vero cells.

**BTV injection of blood mononuclear cells**

The titre of BTV in the supernatant of infected adherent mononuclear cell cultures increased for 72 h after infection, whereas the titre of the cell fraction remained relatively constant for 48 h and then decreased (Fig. 2). No increase in virus titre occurred in the infected cultures of non-adherent cells and the titre of virus in the cell fraction of the non-adherent mononuclear cells was greater than that of the supernatant at all times.

**Fluorescence**

Adherent blood cells present on chamber slides after 24 h of culture, and repeated washing, were mononuclear and a significant proportion of these were readily identified as macrophages on the basis of their size, abundant foamy cytoplasm, and indented nuclei (Fig. 3). At 24 h p.i., 10 to 20% of all adherent mononuclear cells in BTV-infected cultures contained considerable quantities of BTV structural and non-structural proteins, as determined by indirect immunofluorescence staining using MAbs to either NS2 or VP7 (Fig. 4). The fluorescent cells typically had abundant cytoplasm and, often, prominent cytoplasmic processes. Adherent mononuclear cells stained with the MAb specific for VP7 had diffuse, intense cytoplasmic fluorescence with occasional discrete spherical fluorescent inclusions. Cytoplasmic fluorescence in adherent cells stained with the MAb specific for NS2 was more granular, and large discrete fluorescent globules were often present. The pattern of fluorescence obtained with each MAb on infected adherent mononuclear cells was indistinguishable from that obtained when BTV-infected Vero or P3 myeloma cells were stained with the same MAbs. There were fewer fluorescent cells in infected cultures of adherent cells at 44 h p.i. and none at 120 h. The BTV-infected, unstimulated non-adherent cells contained very few cells (approximately 1%) with cytoplasmic fluorescence at 24 h p.i. At 44 and 120 h p.i., even fewer cells were stained and only with the MAb to VP7. Fluorescent cells were not detected in any of the uninfected blood mononuclear cell cultures, nor in BTV-infected cultures of mononuclear cells stained by indirect immunofluorescence using the control MAb.

Double labelling of adherent cells for VP7 and differentiation antigens confirmed BTV infection of monocytes, as BTV-infected mononuclear cells expressed both class II MHC antigen and the class II monocyte-specific antigenic domain recognized by MAb DH59B. A significant proportion of infected cells, however, expressed only small amounts of each differentiation antigen, as determined by indirect immunofluorescent staining with MAb 2D16 and MAb DH59B. The FITC-conjugated MAb recognizing VP7 identified BTV-infected mononuclear cells very specifically, but the intensity of fluorescence was low. Thus, for photography of double-labelled cells (Fig. 5), VP7 was stained by indirect immunofluorescence using the VP7-specific MAb and FITC-conjugated goat anti-mouse IgG, and class II antigen was directly labelled with biotinylated MAb 2D16 and avidin-conjugated Texas Red.
Fig. 1. Autoradiograph of radiolabelled BTV proteins immunoprecipitated from infected BHK-21 cell lysates. Virions were removed by ultracentrifugation from the antigen preparations used in lanes 1, 3, 5 and 6, whereas the preparation used in lanes 2, 4 and 7 was not ultracentrifuged (a cellular protein is present in all these lanes). Lane 1, immunoprecipitation from ultracentrifuged BTV-infected cell lysate using polyclonal rabbit antiserum; lane 2, immunoprecipitation from non-ultracentrifuged BTV-infected cell lysate using polyclonal rabbit antiserum; lane 3, immunoprecipitation from BTV-infected cell lysate by neutralizing MAb specific for VP2; lanes 4, 5 and 6, immunoprecipitation from BTV-infected cell lysates by MAbs specific for NS1, NS2 and VP7, respectively; lane 7, BTV-infected BHK-21 cell lysate without immunoprecipitation. The protein bands are identified on the left of the gel.

Fig. 2. Titres of BTV (log10 TCID50) in the supernatant (○) and cell fraction (●) of (a) adherent and (b) non-adherent cultures of bovine blood mononuclear cells. Cultures were infected at an m.o.i. of 0.02 p.f.u./cell, and harvested at the times indicated.
Fig. 3. Adherent bovine blood mononuclear cells at 24 h after isolation. Most cells have abundant foamy cytoplasm and some have indented nuclei (arrows). Cells were stained with a modified Wright's stain. Bar marker represents 10 μm.
Radiolabelling

Several cellular proteins were less intensely labelled in lysates of cultures of BTV-infected adherent mononuclear cells than they were in lysates of similar cultures of mock-infected cells. Proteins that comigrated with NS2 and VP7 in the infected BHK-21 preparations were present in lysates of BTV-infected adherent mononuclear cells. Lysates of both infected and mock-infected cultures of non-adherent cells contained very few labelled protein bands. The presence of BTV proteins in lysates of the different cultures of bovine mononuclear cells was more readily evaluated by immune precipitation of viral proteins with the polyclonal rabbit antiserum to BTV (Fig. 6). Several BTV-specific proteins, including VP6, VP7, NS1 and NS2 were precipitated from lysates of BTV-infected adherent cells. In contrast, no viral proteins were precipitated from lysates of similar numbers of BTV-infected, unstimulated non-adherent blood mononuclear cells.

Flow cytometry

*In vitro* infection of blood mononuclear cells with BTV was confirmed by FACS analysis. Cultures of BTV-infected adherent mononuclear cells contained greater numbers of infected cells than did cultures of non-adherent cells, as 10 to 17% of all adherent cells reacted with the BTV-specific MAbs, whereas only 2 to 3% of non-adherent cells reacted with the same MAbs (Table 1).
Fig. 6. Autoradiograph of radiolabelled (12 h p.i.) BTV proteins immunoprecipitated from lysates of infected or mock-infected cultures of BHK-21 cells and adherent and non-adherent bovine blood mononuclear cells. Polyclonal rabbit antiserum was used for immunoprecipitation in all lanes. Lane 1, immunoprecipitation from BTV-infected BHK-21 cell lysate; lane 2, immunoprecipitation from uninfected BHK-21 cell lysate; lane 3, immunoprecipitation from BTV-infected adherent mononuclear cell culture lysate; lane 4, immunoprecipitation from uninfected adherent mononuclear cell culture lysate; lane 5, immunoprecipitation from BTV-infected non-adherent mononuclear cell culture lysate; lane 6, immunoprecipitation from uninfected non-adherent mononuclear cell culture lysate. The protein bands are identified at the left of the gel.

The scatter characteristics of monocytes in adherent and non-adherent cultures of blood mononuclear cells were defined by FACS analysis with MAb DH59B, which is specific for bovine monocytes that express class II MHC antigen. Some 82% of all cells in the adherent mononuclear cell cultures reacted with MAb DH59B, whereas only 9% of non-adherent cells
Fig. 7. Dot plots of cultured bovine blood mononuclear cells. All plots reflect FACS analysis of approximately $1.5 \times 10^4$ cells for fluorescence (vertical axis) and granularity, as determined by 90° (side angle) scatter (horizontal axis). Adherent mononuclear cells were stained by indirect immunofluorescence with (a) MAb DH59B specific for bovine monocytes that express class II MHC antigen or (b) with no primary antibody. Non-adherent cells were stained by indirect immunofluorescence with (c) MAb DH59B and (d) with no primary antibody. In all plots, the vertical lines delineate side (90°) scatter channel 7, which is referred to in the text.

Table 1. Percentages of adherent and non-adherent bovine blood mononuclear cells containing BTV proteins at 18 h p.i. as determined by FACS analysis with BTV protein-specific MAbs

<table>
<thead>
<tr>
<th>BTV protein</th>
<th>Adherent cells (%)</th>
<th>Non-adherent cells (%)</th>
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<tbody>
<tr>
<td>VP2</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>VP7</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>NS1</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>NS2*</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>None*</td>
<td>1</td>
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*Percentage of positive cells as identified by FACS analysis with MAb OX-19, specific for rat thymocytes.

reacted with the same MAb (Fig. 7). Furthermore, 98% of the adherent cells and 87% of the non-adherent cells which reacted with MAb DH59B fell in side angle scatter channels of greater than 7. Virtually all BTV-infected mononuclear cells, regardless of whether they were in adherent or non-adherent mononuclear cell cultures, had the same scatter characteristics (side scatter
Fig. 8. Fluorescence histograms of monocytes in BTV-infected cultures of adherent mononuclear cells. Data were collected only from cells gated from the total population of adherent mononuclear cells on the basis of their scatter characteristics (side scatter channels greater than 7). Cells were stained by indirect immunofluorescence with MAbs specific for (a) VP2, (b) VP7, (c) NS1 and (d) NS2. Fluorescence of greater intensity than that indicated by the arrow is considered significant (above background) staining.

Fig. 9. Fluorescence histograms of lymphocytes in BTV-infected cultures of non-adherent mononuclear cells. Data were collected only from cells gated from the total population of non-adherent mononuclear cells on the basis of their scatter characteristics (side scatter channels less than 7). Cells were stained by indirect immunofluorescence using MAbs specific for (a) VP2, (b) VP7, (c) NS1 and (d) NS2. Fluorescence of greater intensity than that indicated by the arrow is considered significant (above background).

channels greater than 7) as the mononuclear cells that reacted with MAb DH59B. The population of BTV-infected cells identified as monocytes on the basis of their scatter characteristics was distinct and exhibited very high mean fluorescence with all BTV-specific MAbs (Fig. 8), especially with the MAb specific for NS1.

Strongly positive cells were considerably less common in cultures of non-adherent cells than they were in cultures of adherent cells, and most strongly positive non-adherent cells had scatter characteristics indistinguishable from those of monocytes. In contrast, although BTV proteins were detected in some cells with scatter characteristics typical of resting lymphocytes (side scatter less than channel 7), intensity of fluorescence of such cells was only slightly above background and most of these cells reacted only with the MAbs specific for structural BTV proteins, and not with MAbs specific for non-structural viral proteins (Fig. 9).

Fluorescence of uninfected adherent and non-adherent blood mononuclear cells reacted with the BTV-specific MAbs was comparable to the background levels of fluorescence obtained when infected cells were reacted with the control MAb (OX-19).

**Electron microscopy**

Virus-like particles and tubules were identified within the cytoplasm of some adherent BTV-infected blood mononuclear cells at 12 h after infection. Tubules (Fig. 10) were morphologically indistinguishable from those described in BTV-infected BHK-21 cells (Huismans & Els, 1979; Eaton et al., 1987). Cells that contained tubules had considerable cytoplasm and abundant
mitochondria, and some had indented nuclei. Cultures of infected adherent mononuclear cells fixed at 18 h after infection contained few intact cells and much cellular debris, in which there were considerable quantities of BTV tubules, and some virus particles (Fig. 11). Virus particles or tubules were not identified within the cytoplasm of cells characterized as unstimulated lymphocytes on the basis of their ultrastructural morphology, nor in cells from uninfected cultures of bovine blood mononuclear cells.

**BTV-infected calf**

BTV was isolated from whole blood collected from the inoculated calf until 35 days p.i. Virus was isolated from mononuclear cells at 1 week after infection, but not thereafter. BTV was isolated from the mononuclear cell fraction only when numbers of cells greater than those present in 1 ml of whole blood were assayed. In contrast, BTV was isolated from erythrocytes until 35 days after inoculation. Late in the course of infection (21 to 35 days after inoculation), BTV was isolated from erythrocytes only when lysates of large numbers of red blood cells ($5 \times 10^8$ to $1 \times 10^{10}$), equivalent to numbers present in 0.1 to 1.0 ml of whole blood, were assayed. In addition, BTV was not isolated from either granulocyte or platelet-enriched cell fractions derived from blood collected at 21 days after inoculation.

Infection of peripheral blood mononuclear cells during the initial phase of viraemia was also confirmed by immunofluorescence staining of mononuclear cells collected from the infected calf. Of approximately $2.5 \times 10^5$ mononuclear cells examined at 1 week after infection, eight
cells were stained with the MAb specific for VP7 and four were stained with the MAb specific for NS2. At 9 days after infection, one cell was stained with the MAb specific for VP7. Fluorescent cells were not detected in preparations containing numbers of erythrocytes similar to those in the mononuclear cell preparations, nor were infected cells identified in stained whole blood smears. Fluorescent cells were not identified in any cell preparations derived from the infected calf at 2 weeks after infection or thereafter. Positive cells were never identified in any samples collected from the control calf.

**DISCUSSION**

A panel of MAbs specific for BTV structural and non-structural proteins was developed and characterized. All MAbs sensitively and specifically identified BTV-infected cells, as assessed by flow cytometry and immunofluorescence staining.

*In vitro* studies with bovine blood mononuclear cells clearly indicate that BTV can infect such cells and that BTV proteins are produced in infected monocytes. Viral proteins were detected in cultures of BTV-infected adherent blood mononuclear cells by immune precipitation of [35S]methionine-labelled proteins from infected cell lysates, indirect immunofluorescence staining of these cells using MAbs specific for structural (VP7) and non-structural (NS2) BTV proteins, and by FACS analysis of infected mononuclear cells using MAbs to VP2, VP7, NS1 and NS2. FACS analysis with the four BTV-specific MAbs indicated that, at the m.o.i. used, up to 17% of all adherent cells contained abundant BTV structural and non-structural proteins at 18 h p.i.

Initial identification of cultured mononuclear cells as monocytes was based upon their morphology, and scatter characteristics as determined by FACS analysis. It was subsequently shown that virtually all adherent cells gated from the total population on the basis of these scatter characteristics (greater than side scatter channel 7) reacted with the MAb specific for bovine monocytes that express class II MHC antigen; although the antigenic domain recognized by MAb DH59B is also present on granulocytes, they were removed during the preparation of mononuclear cell cultures. Double labelling of adherent cells further confirmed that monocytes were infected, as BTV-infected cells expressed both class II MHC antigen and the domain recognized by MAb DH59B. Although class II MHC antigen is also present on bovine B cells and T cell blasts (Baldwin *et al.*, 1988), the domain recognized by MAb DH59B is present only on mononuclear phagocytic cells and granulocytes (Davis *et al.*, 1987). The identity of BTV-infected cells in the adherent population was also confirmed by ultrastructural examination, as BTV tubules, which are composed predominantly of NS1 (Huismans & Els, 1979), and virions were identified only within the cytoplasm of BTV-infected, adherent mononuclear cells with morphological characteristics typical of monocytes.

Despite production of BTV proteins in infected bovine monocytes there was only a minimal increase in the titre of BTV in infected cultures of adherent mononuclear cells, which suggests that monocytes are not especially conducive to productive replication of BTV, at least under the conditions used in these *in vitro* studies. Monocytes and macrophages are inherently resistant to replication of many viruses. They serve as non-permissive cells which phagocytize viruses and so prevent infection of other cells (Morahan & Morse, 1979; Unanue & Allen, 1987). Abortive viral infections of macrophages, characterized by initiation of synthesis of viral proteins but not production of progeny virions, have been described (Nowakowski, 1985; Natuk & Holowczak, 1985). Non-productive infection of monocytes *in vitro*, coupled with the transient presence of BTV-infected mononuclear cells in the peripheral blood of the BTV-infected calf, indicates that persistent infection of monocytes is unlikely to be responsible for the prolonged viraemia that occurs in BTV-infected cattle. These results are compatible with our previous observation that persistent infection of blood mononuclear cells does not occur in calves experimentally infected with this strain of BTV (MacLachlan & Fuller, 1986).

Although most BTV-infected adherent mononuclear cells were monocytes, these *in vitro* studies did not establish whether or not blood mononuclear cells other than monocytes can be infected with BTV. Minimal replication of BTV occurred in cultures of non-adherent blood mononuclear cells, confirming previous observations that viruses are unlikely to replicate in
BTV infection of bovine monocytes

resting lymphocytes (Doyle & Oldstone, 1979). The few strongly positive cells in BTV-infected cultures of non-adherent cells had scatter characteristics indistinguishable from those of monocytes. Adherence removes only a portion of the total population of monocytes present in isolated blood mononuclear cells (Pennline, 1981), and significant numbers of monocytes were demonstrated in the non-adherent cultures by FACS analysis with monocyte-specific MAb DH59B. However, it was not established whether all strongly positive cells in the non-adherent cultures were monocytes, as a significant proportion of the cells with the same scatter characteristics as monocytes did not react with MAb DH59B. Thus, while our results clearly indicate that BTV replicates in blood monocytes and not unstimulated lymphocytes, double labelling of BTV-infected blood mononuclear cells with a panel of well characterized MAbs to bovine leukocyte differentiation antigens, together with MAbs to BTV proteins, will be necessary to determine whether replication can occur in stimulated lymphocytes or any other blood cell population.

The mechanism responsible for infection of blood cells in BTV-infected cattle is not determined. It is obvious, however, that although BTV initially was associated with both mononuclear cells and erythrocytes in the blood of the infected calf, late in the course of infection virus was consistently associated with only erythrocytes. Furthermore, the concentration of virus in the erythrocyte fraction is similar to that in whole blood, whereas virus titres in the mononuclear fraction are lower than those in whole blood. These results support the observations of Luedke (1987), and contrast with those of Collisson & Barber (1983) and Parsonson et al. (1970). Conflicting results obtained by different investigators most likely reflect differences in the numbers of cells evaluated in each individual blood cell population. In addition, the different routes of inoculation of cattle and unique properties of the different BTV serotypes and strains used in these contradictory studies might influence cellular association during viraemia.

Our failure to demonstrate BTV-infected erythrocytes by immunofluorescence staining was not unexpected because infected red cells are extremely uncommon in the blood of infected cattle; late in the course of infection, for instance, virus was isolated from red cell fractions only when numbers of erythrocytes comparable to those in 0.1 to 1.0 ml of blood were assayed. It is also unknown whether infected erythrocytes carry sufficient viral antigen to permit detection by indirect immunofluorescent staining. At least two plausible explanations could be proposed to account for infection of erythrocytes in BTV-infected cattle. First, BTV may infect erythroid stem cells in the bone marrow during initial viraemia. Maturation of infected erythroid precursors into red cells might prevent or delay virus-mediated lysis of the infected cell, and virus could then circulate within infected red cells for prolonged periods. A similar hypothesis has been proposed for the prolonged, cell-associated viraemia that occurs in Colorado tick fever virus-infected mammals (Emmons et al., 1972). Secondly, it is possible that virus is released after lysis of BTV-infected endothelial cells and immediately adsorbs to circulating blood cells. In this case, red cells would best be considered as passive carriers of the virus. Immunohistochemical staining of bone marrow taken from calves at various intervals after BTV infection is currently being undertaken in our laboratory, in an effort to characterize more accurately the mechanism responsible for infection of erythrocytes in BTV-infected cattle.

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REFERENCES


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