Interaction of bluetongue virus with bovine lymphocytes

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Freshly isolated, and established, cultures of bovine peripheral blood mononuclear leukocytes (PBMLs) were exposed to bluetongue virus (BTV) for the purpose of defining potential lymphotropism. PBML cultures were established in the presence of interleukin 2 (IL-2) and mitogen and maintained either as bulk culture or were cloned prior to infectivity studies. All cultures appeared to be of the T cell phenotype based on the following characteristics: binding of T lymphocyte-specific lectins (i.e. peanut agglutinin and Helix pomatia), rosetting of sheep erythrocytes, binding of a putative pan-T monoclonal antibody, and absence of surface immunoglobulin (Ig). T lymphocyte cultures were further characterized by their ability to elicit lectin-dependent cellular cytotoxicity (LDCC). Exposure of established lymphocyte cultures to BTV resulted in productive cytopathic and non-cytopathic infections. Non-cytopathic productive infections were observed in LDCC-negative cultures whereas cytopathic and non-cytopathic infections were observed in LDCC-positive cultures. Exposure of freshly isolated PBMLs to BTV resulted in minimal virus replication; addition of mitogen and IL-2 to such cultures did not augment virus replication. Addition of mitogen and IL-2 induced negligible blast transformation, whereas PBML viability was minimally affected. These studies establish a tropism of BTV for bovine T lymphocytes with virus replication being limited to those cells undergoing blastogenesis. Establishment of infected lymphocyte cultures, without loss of culture viability, suggest such an interaction may contribute to the long term viraemias associated with BTV infection of cattle.

Introduction

Pathogenesis of bluetongue virus (BTV) infection in cattle is poorly documented, particularly its relationship to cell tropism. Although BTV-infected cattle rarely express clinical disease, the infections are characterized by establishment of long term viraemias (often in excess of 60 days) in the presence of high titre neutralizing antibody (DuToit, 1962; Heidner et al., 1988; Luedke et al., 1969; MacLachlan & Fuller, 1986; MacLachlan et al., 1987; Richards et al., 1988). Such coexistence of virus and antibody suggests a sequestered association with cells. Studies by Luedke (1970) suggested a preferential association of virus with erythrocytes although virus was also isolated from buffy coats. Whetter et al. (1989) similarly demonstrated preferential association of virus with erythrocytes although virus was isolated from peripheral blood mononuclear leukocytes (PBMLs) at 1 week post-infection. Parsonson et al. (1987) suggested non-preferential association of virus with erythrocytes and buffy coats whereas Collisson & Barber (1983) reported similar virus titres in all blood cell fractions (erythrocytes, mononuclear leukocytes and polymorphonuclear leukocytes). Electron microscopy of peripheral blood, obtained from BTV-infected cattle, has demonstrated virus-like particles associated predominantly with monocytes and less so with lymphocytes (Morrill & McConnell, 1985). Regardless of the cell population(s) with which the virus is associated, the prolonged viraemias are probably due to virus being inaccessible to neutralizing antibody.

The isolation of virus from a given peripheral blood cell fraction does not provide information related to the production of progeny virus. This is best illustrated by the isolation of virus from erythrocytes, an association which would not be compatible with virus replication except at the progenitor stage in the bone marrow. The intent of this study was to determine whether in vitro cultures of bovine lymphocytes could be productively infected with BTV. Similar studies have recently demonstrated infection of bovine monocytes although minimal virus progeny were produced (Whetter et al., 1989).

Methods

Virus. BTV serotype 17 (strain 62-45-S) was used in this study. Virus stock was propagated on confluent monolayers of BHK-21 cells until c.p.e. reached 95%. Cells and supernatant were harvested, sonicated
and cellular debris was removed by centrifugation at 2000 g. The supernatant was diluted with an equal volume of preservative (buffered lactose peptone) (Howell et al., 1967) and frozen at -70 °C. Virus stocks were titrated by serial 10-fold dilutions and applied to confluent monolayers of African green monkey kidney (Vero-Maru) cells in 24-well cluster plates (Stott et al., 1978). Following adsorption for 1 h at 37 °C, cells were overlaid with 0.5% agarose in MEM containing 5% foetal bovine serum (FBS). Forty-eight h later, cells received a second overlay containing 0.08% neutral red. Plaques were counted 4 days after the second overlay.

**Lymphocyte cultures.** Bovine PBML cultures were established as previously described (Scibienski et al., 1987). PBMLs were isolated on Ficoll-Hypaque, washed and adjusted to a cell concentration of 10^6 cells/ml in RPMI medium containing 10% FBS. Lymphocyte cultures were established using human recombinant interleukin-2 (IL-2) which was kindly provided by the Cetus Corporation. IL-2 was used at a concentration of 10 units/ml in growth medium. In addition to IL-2, one of three mitogens was added to the growth media as follows: concanavalin A (Con A) at 0.5 μg/ml, pokeweed mitogen at 0.5% or phytohaemagglutinin at 1.0 μg/ml. Lymphocyte cultures were maintained by periodic splitting of cultures as dictated by cell density. Lymphocyte clones were established by limiting dilution onto feeder cells using fresh IL-2 and mitogen in conditioned medium. Conditioned medium and mitomycin C-treated feeder cells were obtained from the same bulk cultures as used for cloning. An established bovine leukaemic cell line (BL-3), obtained from G. Theilen (University of California, Davis, Ca., U.S.A.) was also used in this study (Theilen et al., 1982). This latter lymphocyte line was maintained without exogenously derived growth factors.

**Lymphocyte characterization.** Lymphocyte cultures were tested for IL-2 dependence by removal of the lymphokine. All cultures were phenotypically and functionally characterized as follows. Fluorescence microscopy was done for the detection of cell-surface binding of fluorescein isothiocyanate-conjugated peanut agglutinin (PNA), *Helix pomatia* lectin (HP), anti-bovine surface immunoglobulin (Ig), and a putative bovine pan-T monoclonal antibody (MAb) 171.H5 (Scibienski et al., 1987). Also, sheep erythrocyte rosetting (Paul et al., 1979), and ability to elicit lectin-dependent cellular cytotoxicity were investigated (LDCC) (Scibienski et al., 1987).

**Virus infection of lymphocytes.** Lymphocytes were washed in RPMI and adjusted to 2 x 10^6 cells/ml. Viability was determined by trypan blue exclusion. Virus was diluted in RPMI to a titre of 2 x 10^6 p.f.u./ml. Equal volumes of virus and cell suspensions were mixed, such that an m.o.i. of 1.0 was obtained, and incubated on a rocker platform at 37 °C for 1 h. Following incubation, cells were washed free of residual virus, resuspended in complete growth medium containing IL-2 and mitogen, and cultured for 6 days in 24-well cluster plates. Cell viability and virus titre were determined at a minimum of 24 h intervals with zero time point sample being taken immediately upon removal of residual virus after virus adsorption. All samples were held at 4 °C until the experiment was terminated, at which time virus titration was conducted.

**Results**

**Establishment and characterization of lymphocyte cultures**

Conditions for establishment, maintenance and cloning of bovine T lymphocyte cultures have previously been described (Scibienski et al., 1987). All established cultures were dependent upon IL-2 supplied exogenously. Lymphocyte cultures were maintained for a minimum of 1 month prior to characterization and/or infection with BTV. All lymphocyte cultures appeared to be of the T cell lineage as determined by erythrocyte rosetting, binding of the lectins PNA and HP, and binding of the putative pan-T cell MAb 171.H5; the BL-3 clone was positive only when tested with 171.H5 and did not bind lectins or sheep erythrocytes. All cultures were devoid of cells expressing surface IgG. Five of the six established lymphocyte cultures were tested for ability to elicit LDCC; two of these have been described previously (1991 PW clone and the 618 B01 clone) (Scibienski et al., 1987). Two cultures (the BL-3 clone and the 1891 PW clone) were LDCC-negative and three (the 1028 bulk culture, the 1031 bulk culture and the 618 B01 clone) were LDCC-positive; the 723 bulk culture was not tested.

**Infection of established lymphocyte cultures**

BTV-infected cultures were harvested for virus titration at a minimum of 24 h intervals. The first sample, at zero time, represented cells which had been washed immediately following virus adsorption (Fig. 1, 2 and 3). The virus control (Fig. 1a) represented virus added to culture plates in the absence of cells to control for persistence of non-replicating virus in the growth medium, and loss of titre over the 6-day storage period prior to virus titration. As illustrated in Fig. 1(a), the control virus preparation gradually lost titre over the incubation period with less than 100 p.f.u./ml being present on day 6. Following adsorption, an immediate decrease in virus titre was recorded for the LDCC-negative cultures and for the bulk culture for which LDCC status was not determined (Fig. 1b to d). These three cultures produced titres in excess of 10^6 p.f.u./ml by 24 to 48 h and then remained constant or decreased in titre until day 6. Cell viability was variable with the 723 bulk culture and BL-3 clone undergoing a cytopathic infection and for the 1891 clone undergoing a non-cytopathic infection.

Infection of the three LDCC-positive lymphocyte cultures (1028 bulk, 1031 bulk and 618 B01 clone) resulted in productive non-cytopathic infections (Fig. 2). As described for the LDCC-negative cultures, immediate decreases in virus titre were observed after virus adsorption followed by an increase in titre which peaked at 96 h. Cell viability remained generally constant in 1028 bulk and 618 B01 cultures. Viability of the 1031 bulk culture decreased until 72 h and then increased until 96 h, to exceed 60%.

**Infection of freshly isolated peripheral blood mononuclear cells**

PBMLs derived from two animals (no. 2 and no. 4) were exposed to virus and placed in culture media with and
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Fig. 1. Titre (p.f.u./ml) of virus (BTV strain 17) in growth media with no cells (a). Virus titre (▲; p.f.u./ml) and cell viability (●; trypan blue exclusion) of established lymphocyte cultures (b, c and d) over time. BL-3 and 1891 PW clones (c and d, respectively) were LDCC-negative; the 723 bulk culture (b) was not tested. Cultures were infected at an m.o.i. of 1.0 and virus titre was determined at the times indicated. Time 0 represents cells that had been washed immediately following virus adsorption.

Fig. 2. Virus titre (▲; p.f.u./ml) and cell viability (●; trypan blue exclusion) of established lymphocyte cultures over time. (a) Bulk culture 1028; (b) bulk culture 1031; (c) clone 618 B01. All cultures exhibited LDCC activity. Cultures were infected at an m.o.i. of 1.0 and virus titre was determined at the times indicated. Time 0 represents cells that were washed immediately following virus adsorption.

without IL-2 and Con A (Fig. 3). Virus titre dropped gradually over the 6 day culture period. Cell viability remained unaltered in PBMLs derived from animal no. 2 (Fig. 3a, b) while a decrease approaching 50% was recorded for PBMLs from animal no. 4 (Fig. 3c, d). Blast formation, as determined by microscopic observation, was minimal in all cultures; cell size remained small and the few colonies that developed were limited to two or three cells. This was in contrast to cells not receiving virus, which developed into large blasting lymphocytes, the majority of which were associated in large colonies (greater than five cells/colony).
Discussion

Lymphocyte cultures employed in these studies represented either freshly isolated PBMLs or blasting IL-2-dependent cultures which had been propagated for a minimum of 30 days. Cloned lymphocyte lines were obtained by limiting dilution followed by an additional 30 days in culture to obtain sufficient cell expansion for infectivity studies. Such long term cultures were phenotypically characterized as being of the T lymphocyte lineage by cell-surface binding of the T lymphocyte-specific lectins PNA and HP (Morein et al., 1979; Johansson & Morein, 1983; Pearson et al., 1979), rosetting of sheep erythrocytes (Paul et al., 1979) and a putative pan-T MAb (Scibienski et al., 1987); none of the cultures expressed surface Ig as determined by fluorescence microscopy. T lymphocyte cultures were characterized further by their ability to elicit LDCC; three cultures were positive and one was negative. The LDCC-negative cells are assumed to be of the T helper/inducer subpopulation, and the positive cells of the T cytotoxic/suppressor subset; however such a contention is merely speculative. An additional cell culture employed was an established growth factor-independent mononuclear leukocyte line previously obtained from a cow with leukaemia (Theilen et al., 1982). We assume tentatively this cell line is of the T lymphocyte lineage based upon absence of surface Ig and binding of our pan-T MAb. However, this line did not bind PNA, HP or sheep erythrocytes and could potentially represent a non-T, non-B lymphocyte.

Productive BTV infections were established in all lymphocyte cultures, regardless of the functional LDCC subset (Fig. 1 and 2). Variability in the virus titre at time zero (immediately following virus adsorption and washing) was observed between cultures (Figs. 1, 2 and 3). We assume this variability was due to efficiency of virus adsorption. The relatively low titre recorded for the virus control (Fig. 1 a) was not unexpected as all samples were held at 4 °C for a minimum of 1 week prior to virus titration; stability of BTV infectivity is greatly reduced in the absence of protein or other stabilizing solutions.

Lymphocyte viability was a variable feature with infection of the BL-3 clone and the 723 bulk culture being cytopathic. Onset of c.p.e. was very rapid in the BL-3 cells with a few viable cells being left at 30 h. Cell death was more gradual in the 723 bulk culture and 120 h was required for 90% cell death. In contrast, infection of all other lymphocytes did not result in culture death and at 6 days post-infection cell viability ranged from 50 to near 100%. Two such bulk cultures (1891 and 1031) experienced some early cell death but recovered to pre-infection status through cell replication by 96 h. The mechanism(s) underlying these latter non-cytopathic

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Fig. 3. Virus titre (▲; p.f.u./ml) and cell viability (●; trypan blue exclusion) over time, of freshly isolated PBMLs from animal no. 2 (a and b) and no. 4 (c and d). Cells were exposed to virus in the presence (a and c) and absence (b and d) of Con A (mitogen) and IL-2. Cultures were infected at an m.o.i. of 1.0 and virus titre was determined at the times indicated. Time 0 represents cells which were washed immediately after virus adsorption.
infections are uncertain. As all cells in the infected cultures were not expressing viral antigen, as determined by fluorescence microscopy, we could not determine whether individual cells were persistently infected or whether replication of non-infected cells provided a continual source of target for the progeny virus slowly being released from cells infected cytopathically.

Upon establishment that blasting T lymphocytes could be infected productively with BTV, viral interaction with resting lymphocytes was studied (Fig. 3). Freshly isolated PBMLs were exposed to virus and then placed in culture with and without mitogen and IL-2. Although virus adsorbed readily to the lymphocytes, minimal virus replication occurred in relation to that observed in the blasting cells. Virus titre decreased gradually in cells derived from animal no. 4 as did lymphocyte viability. Virus titre remained relatively constant in cells from animal no. 2 and lymphocyte viability decreased only modestly. Addition of mitogen to either culture had a dramatic effect on neither virus titre nor on cell viability. We would conclude from these studies that resting lymphocytes can support little virus replication. The absence of a drop in virus titre over time in PBMLs from animal no. 2 could be due to virus replication in monocytes, which can support minimal virus production (Whetter et al., 1989), or the virus may have been protected from degradation through its association with viable cells. The latter hypothesis may be more likely as the cultures with the greatest drop in cell viability (no. 4) had a concurrent drop in virus titre. Regardless of culture viability, exposure to virus resulted in lymphocytes being non-responsive to mitogen and IL-2 as minimal blast formation was observed.

The relevance of these in vitro studies to BTV pathogenesis in cattle is yet to be determined. In relation to cell tropism, we feel confident that the lymphocytes established in culture closely resemble those in vivo since cell phenotype and cytolytic activity remained unaltered. We speculate that exposure of blasting lymphocytes to BTV in cattle would result in a productive infection and contribute to virus replication and possible dissemination throughout the body. Interaction of virus with resting lymphocytes would not appear to have the potential for production of progeny virus but could facilitate virion survival by stabilization of an infectious particle.

Association of BTV–lymphocyte interactions with the long term viraemias typically observed in cattle will require further studies. The studies reported here establish the lymphotropic nature of the virus and pose the following questions. Does association of virus with resting lymphocytes serve to sequester the infectious virion away from neutralizing antibody and contribute to virus dissemination? Secondly, does association of virus with resting lymphocytes contribute to prolonged viraemia by interfering with blast transformation and expansion of BTV-specific lymphocytes? Thirdly, can infection of blasting lymphocytes result in non-cytopathic productive cellular infections with subsequent long term release of infectious virus into the blood, via lymph?

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