Original Article

The Contribution of Bovine Leukaemia Virus Infected B-cells to the Number of Circulating B-cells in Cattle

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Abstract. This study used a combination of single antigen immunoperoxidase staining for bovine leukaemia virus (BLV) p24 capsid antigen and IgM to enumerate the peripheral blood mononuclear cells (PBMCs) infected with BLV and the number of B-cells, respectively. A significant relationship was found between the number of BLV-infected PBMCs and the number of circulating B-cells. A model was created that predicted the number of circulating B-cells using the number of BLV-infected PBMCs. These data also show that the percentage of B-cells in PBMC preparations is not affected by 24 h of in vitro culture when LPS is added to the culture medium. Double antigen labelling showed that the majority of circulating B-cells were infected with BLV in some BLV-seropositive cattle that were not persistently lymphocytotic.

Keywords: B-cells; BLV-p24 capsid protein; Bovine leukaemia virus; Immunocytochemistry; Persistent lymphocytosis

Introduction

Bovine leukaemia virus (BLV) is the aetiological agent of endemic bovine lymphoma (Miller et al. 1969). The BLV-provirus is present in BLV related tumors, and cellular infection with BLV is necessary for neoplastic transformation (Kettmann et al. 1980a). BLV also causes persistent lymphocytosis (PL) in some infected cattle. In adult cattle 2 years of age or older, PL is defined as a sustained circulating lymphocyte count of at least 10000/µl of blood. Since lymphocyte counts in cattle decrease with age this minimum count decreases with age (Perman et al. 1970; Moulton and Harvey 1990). Most of the lymphocytes associated with lymphocytosis are B-cells (Muscoplat et al. 1974; Weiland and Straus 1975; Paul et al. 1979). BLV-infected cattle, that are not persistently lymphocytotic, may also exhibit increased numbers of circulating B-cells (Fossum et al. 1988; Lewin et al. 1988; Williams et al. 1988a) and phenotypic alteration of some circulating B-cells (Depelchin et al. 1989).

BLV antigens and mRNA for structural proteins are generally not detectable in BLV-infected animals (Ferrer 1980; Kettmann et al. 1980b). Viral expression occurs after culturing peripheral blood mononuclear cells (PBMCs) in vitro (Driscoll and Olson 1977). Phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) enhance viral expression, but are not essential (Driscoll and Olson 1977; Lagarias and Radke 1989). Cultured B-cells and T-cells, purified from PBMCs, have been used to show that B-cells produce BLV antigens and virus (Kenyon and Piper 1977a; Paul et al. 1977; Jensen et al. 1990). However, there is evidence that T-cell lines derived from the PBMCs of BLV-infected cattle are BLV-infected (Stott et al. 1991). There is also evidence that T-cells (Williams et al. 1988b), endothelial cells (Rovnak et al. 1991), and monocytes (Heeney et al. 1992) are infected in vivo. Purified B-cell preparations should contain a greater percentage of BLV-infected cells than paired samples of PBMCs. However, studies have shown that cultured B-cells contain similar percentages of BLV-antigen expressing cells as unfractionated PBMCs from the...
same animal (Jensen et al. 1990), and may produce less infectious virus (Paul et al. 1977). Thus, although it is certain that B-cells produce BLV in vitro, it is not certain that B-cells are the only cells that produce BLV in cultures of unfractionated PBMCs.

Kenyon and Piper (1977b) used density gradient fractionation of PBMCs to separate the B-cells of PL cattle into those that produced BLV in vitro and those that spontaneously incorporated tritiated thymidine. Their results suggested that part of the increases in circulating B-cells was due to B-cells immunoreactive to BLV but not infected with the virus. From this it was postulated that PL is not a proliferation of preneoplastic BLV-infected cells, but is an immunological reaction to BLV infection.

The present study uses single and double antigen immunocytochemistry to determine the relationship between BLV-infected B-cells and circulating B-cells. Surface and cytoplasmic immunoglobulin (Ig) are definitive markers for B-cells. Previous studies have not been able to determine accurately B-cell numbers after culture because B-cells cultured with PHA did not express immunoglobulin (Esteban et al. 1985a,b). The present study used culture methods that preserved or enhanced Ig as a definitive marker for B-cells. Paired samples of PBMCs labelled for BLV-p24 or IgM were used to show a relationship between the number of circulating B-cells and BLV-infected PBMCs in cattle, even in cattle that were not persistently lymphocytotic.

Double immunocytochemistry for immunoglobulin and BLV-p24 capsid antigen was used to show that B-cells are the predominant cell that produces BLV antigen in short-term cultures of unfractionated PBMCs.

Materials and Methods

Animals

Cattle free of BLV infection were from a BLV-free herd maintained at the United States Department of Agriculture, Dairy Forage Research Center, Lodi, WI (Kaja et al. 1984). These cattle were used as controls and to determine the number of circulating B-cells in cattle free of BLV infection. BLV-infected Holstein cattle were from a privately owned herd with a high prevalence of seropositive animals and a high incidence of lymphoma. An agar gel immunodiffusion assay (AGID) (Pitman-Moore, Mundelein, IL), detecting antibodies to BLV-gp51 envelope protein, was used to determine if cattle were infected with BLV. Total white cell counts were performed using an automated cell counter (Coulter Electronics Inc., Hialeah, FL), and differentials were made using blood films stained with Wright's Stain. The absolute lymphocyte counts were calculated from total white cell counts and lymphocyte percentages. Blood for these determinations was collected into vacuum tubes treated with EDTA (Becton Dickinson, Rutherford, NJ).

PBMC

PBMCs were isolated from citrated blood layered over Ficoll–Hypaque as described by Schultz and Adams (1978). PBMCs were cultured for 24 h at $2 \times 10^6$ cells/ml in Dulbecco’s Modified Eagle’s Medium (DMEM) with 4.5 g/l glucose, 110 mg/l sodium pyruvate and $2 \times 10^{-5}$ M-2 mercaptoethanol (Sigma Chemical Company, St Louis). BLV expression was enhanced by addition of 50 μg/ml LPS from Salmonella minnesota or 5 μg/ml PHA (Sigma Chemical Company). Medium was supplemented with 10% fetal bovine serum (FBS) (Sigma Chemical Company), 20% swine serum or 20% rabbit serum (Gibco Laboratories, Grand Island, NY).

Hybridoma and Ascites Production

A hybridoma secreting a monoclonal antibody to BLV-p24 capsid protein was generated using the methods described by Galfré et al. (1977). The myeloma cell line was the P3X63AG8 non-secreting subclone of the Balb/c mouse myeloma NS1 (American Type Culture Collection, Rockville, MD). Balb/c mice were inoculated with BLV derived from spent culture medium from Bat2C16 (Diglio and Ferrer, 1976) concentrated 1000-fold. A hybridoma, identified as Mab7.10.6, was cloned three times by limiting dilution; clonality was confirmed by visual inspection and Poisson distribution (Henry et al. 1980). Clones were confirmed to be secreting BLV-specific antibody by immunocytochemistry on BLV-infected syncytia in bovine embryonic spleen cells (Ferrer and Diglio 1976). The monoclonal antibody was determined to be the isotype IgG1, kappa (Zymed Inc., Camarillo, CA). Immunoblot determined that the monoclonal was specific to BLV-p24 (Towbin et al. 1979). Ascites was collected 10 days to two weeks after mice were inoculated with hybridoma cells.

Immunocytochemistry

PBMCs cultured in FBS were washed three times in Hank’s buffered saline solution. PBMCs cultured with serum from other species were not washed. Cytospin preparations (Shandon Inc., Pittsburgh, PA) of approximately $5 \times 10^5$ PBMCs were made before and after 24 h in culture. Slides were fixed in acetone at 4°C for 5 min.

An avidin–biotin complex technique was used (ABCelite) (Vector Laboratories, Burlingame, CA) for single antigen immunoperoxidase. Ascites containing a monoclonal antibody to ungulate IgM (PIg45a) (VMRD Inc., Pullman, WA) or BLV-p24 (Mab7.10.6) was diluted 1:1000 and used as the primary antibody. Diaminobenzidine (Sigma Chemical Company) was used as the chromogenic substrate. Slides were counterstained with Mayer’s haematoxylin.

The immunoalkaline-phosphatase ABCelite kit (Vector Laboratories) and the peroxidase antiperoxidase technique were used for double immunocytochemistry for BLV-p24 and immunoglobulin. Slides were fixed in acetone at 4°C for 5 min.

The present study uses single and double antigen immunocytochemistry to determine the relationship between BLV-infected B-cells and circulating B-cells. Surface and cytoplasmic immunoglobulin (Ig) are definitive markers for B-cells. Previous studies have not been able to determine accurately B-cell numbers after culture because B-cells cultured with PHA did not express immunoglobulin (Esteban et al. 1985a,b). The present study used culture methods that preserved or enhanced Ig as a definitive marker for B-cells. Paired samples of PBMCs labelled for BLV-p24 or IgM were used to show a relationship between the number of circulating B-cells and BLV-infected PBMCs in cattle, even in cattle that were not persistently lymphocytotic. Double immunocytochemistry for immunoglobulin and BLV-p24 capsid antigen was used to show that B-cells are the predominant cell that produces BLV antigen in short-term cultures of unfractionated PBMCs.