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Cell-mediated cytotoxic responses in lungs following a primary bovine herpes virus type 1 infection

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SUMMARY

Non-major histocompatibility complex (MHC) restricted cytotoxicity is an important part of the immune reaction mounted in response to bovine herpes virus type 1 (BHV-1) infection. In this study, we evaluated the effect of BHV-1 infection on the ability of lung parenchyma leucocytes (LPL), cranial tracheobronchial lymph node cells (BLNC) and peripheral blood mononuclear leucocytes (PBML) to mediate this function. While LPL from non-infected calves mediated cytotoxicity against BHV-1-infected cells, a similar activity could not be detected in PBML or BLNC. In contrast, both LPL and PBML from naive calves could mediate cytotoxicity against K562 target cells but only after activation with interleukin-2 (IL-2). BLNC were unable to kill K562 cells. Infection of calves with BHV-1 enhanced the ability of LPL and PBML to kill BHV-1-infected cells. This enhancement was detected as early as Day 1 after infection in LPL whereas it could only be detected in PBML 8 days after infection. The results demonstrate that the leucocyte population present at the site of infection was able to mediate a potentially important antiviral function and that this function was enhanced rapidly in response to infection. Thus LPL-mediated cytotoxicity may be an important mechanism for the recovery from BHV-1 infection.

INTRODUCTION

Bovine herpes virus 1 (BHV-1) is a common respiratory pathogen of cattle and many investigations exist dealing with the possible immune mechanisms responsible for recovery and protection from this virus. As with other herpes viruses, cell-mediated immune responses are thought to play a primary role in recovery from primary infection with BHV-1 and special attention has been given to cytotoxic mechanisms that may be involved in defence against BHV-1. Mechanisms described include T-cell-mediated antigen-dependent cellular cytotoxicity [mediated by cytotoxic T lymphocytes (CTL)],[1-3] antigen-dependent non-major histocompatibility complex (MHC) restricted cytotoxicity mediated by macrophages,[4] antibody-dependent cellular-cytotoxicity (ADCC) mediated by neutrophils and macrophages,[5,6] and a variety of antigen non-specific non-MHC restricted cytotoxicities mediated by natural killer (NK) cells and lymphokine-activated killer (LAK) cells.[7,8] The most prominent cytotoxic activity detected after primary virus infection has been antigen-dependent, non-MHC restricted cytotoxicity.[9] This activity is mediated by an adherent cell and interferon-gamma (IFN-γ) produced during the cytotoxicity assay is required for cytotoxicity to occur.[4,10] However, these studies used leucocyte populations obtained from peripheral blood, and their involvement in protection of the respiratory tract has not yet been established.

Similar to the skin and the gastrointestinal tract the lung acts as an important interface between the sterile internal environment and the contaminated external environment. Therefore, it can be anticipated that cells involved in the protection and/or recovery from respiratory tract diseases should be situated within the respiratory tract, and/or should be able to infiltrate effectively these areas during respiratory infections.

In human beings and mice, functionally active lung parenchyma leucocytes (LPL) have successfully been obtained through enzymatic digestion of lung tissue.[11,12] This population of cells has been suggested to constitute a localized leucocyte population with intrinsic characteristics of probable relevance to respiratory immunity.[11-14] Since BHV-1 is primarily a respiratory pathogen we investigated the effect of BHV-1 infection on the cytotoxic activity of LPL and compared this activity with that of leucocytes obtained from peripheral blood and regional lymph nodes.

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; BHV-1, bovine herpes virus type 1; BLNC, bronchial lymph node cells; CTL, cytotoxic T lymphocytes; LAK, lymphokine-activated killer; LPL, lung parenchyma leucocytes; MHC, major histocompatibility complex; NK, natural killer; PBML, peripheral blood mononuclear leucocytes.

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MATERIALS AND METHODS

Virus stock

BHV-1 (strain 108) grown in GBK cells at a titre of 4×10^7 PFU/ml was used for infection of experimental animals as well as for infection of cells for *in vitro* cytotoxicity assays.

Tissue culture conditions and reagents

Modified Eagle's minimum essential medium (MEM) was supplemented with 50 μg/ml of gentamycin (both from Gibco Laboratories, Grand Island, NY), 2 mg/ml of sodium bicarbonate (Fisher Scientific Co., Fair Lawn, NJ), 25 mM HEPES (BDH Chemicals, Toronto, Canada), and 10% foetal bovine serum (Gibco) was used throughout these experiments. All tissue culture incubations were carried out at 37° in a humidified atmosphere of 95% air and 5% CO_2.

Animals and experimental protocol

Twenty-one Hereford beef calves, 4 months of age and seronegative to BHV-1, were used for this study. Eighteen calves were experimentally infected with BHV-1 by an aerosol challenge as previously described.[15] Three calves served as non-infected controls. On Days 1, 2, 3, 4, 5, and 8 post-infection three randomly chosen infected calves were killed. Calves were treated in accordance with the guidelines for experimental animals established by the Canadian Council on Animal Care. Blood samples were collected prior to infection and just before killing from all calves. Complete post-mortem examinations were performed on all calves and tissue samples were collected from the lungs and the cranial tracheobronchial lymph nodes for leucocyte isolation.

Preparation of effector cells

Lung parenchyma leucocytes were obtained from lungs of both BVH-1-infected and non-infected calves using a modification of the method described by Holt *et al.*[11] Lung samples (approximately 5 cm^3) were dissected free of capsule and minced finely using a scalpel. Minced tissue was placed in 100-ml bottles containing 50 ml volumes of MEM supplemented with 15% FBS and 50 U/ml DNase (medium 1). Batches of 10 g of tissue were processed in a single bottle. These bottles were placed on a shaking platform at 37° with 120 oscillations/min. After 20 min incubation, the medium was removed and replaced with 50 ml of fresh medium 1. The incubation with shaking was repeated to remove the majority of occluded blood cells in the tissue. Washed tissue was blotted dry using sterile paper towels and placed in clean bottles (approximately 5 g of tissue per bottle) containing 50 ml medium 1 supplemented with 300 U/ml of collagenase. Bottles were incubated (with shaking) for 1 hr at 37°. Following digestion, the mixture was sieved through a mesh and filtered through a sterile gauze. Recovered cells were washed free of collagenase and DNase by centrifugation. The cell pellet recovered from each bottle was resuspended in 35 ml of Hanks' Balanced Salt Solution (HBSS) and layered on 15 ml of Ficoll-Paque (specific gravity 1·077 g/ml, Pharmacia LKB Biotechnology Inc. Piscataway, NJ). After centrifugation at 800 ***g*** for 45 min, cells were recovered from the interface and washed twice in HBSS. To obtain bronchial lymph node cells (BLNC), lymph nodes were cut in small pieces and sieved through a stainless steel mesh. The recovered cell suspension was washed by centrifugation and separated on a Ficoll-Paque gradient as described for LPL. PBML were obtained as described in detail elsewhere.[4] Briefly, peripheral blood was collected into citrate dextrose and the buffy coat was recovered by centrifugation. PBML were isolated from buffy coat cells on Ficoll-Paque and washed three times in HBSS. All cell populations were resuspended at 1×10^7 cells/ml for cytotoxicity assays and at 2×10^7 cells/ml for flow cytometry. Viability in all cell populations was greater than 95% by trypan blue exclusion.

Cytotoxicity assay

To study the cytotoxic capability of effector cells against BHV-1-infected and non-infected cells a ^{51}Cr release assay using GBK cells as target cells was performed as previously described.[7] Briefly, after overnight labelling of GBK cells with $Na_2\ ^{51}Cr\ O_4$ (Amersham Canada Ltd, Oakville, Ontario) and 2 hr infection with BHV-1, effector cells were added at a 100:1 effector:target cell ratio. The ability of effector cells to mediate natural killer (NK) cell activity against K562 target cells was assessed using a standard ^{51}Cr release assay. After labelling with $Na_2{}^{51}Cr\ O_4$ for 2–3 hr, K562 cells were washed and effector cells were added at a 100:1 effector:target ratio. This assay was also performed in the presence of IL-2 (100 U/ml recombinant human IL-2, Boerhinger-Mannheim, Mississauga, ON) to assess the ability of effector cell populations to mediate LAK cell activity. Maximum release of ^{51}Cr was assessed by culturing target cells in 2·5% Triton X-100 (Sigma, St Louis, MO) and spontaneous release by culturing target cells in medium alone. The percentage cytotoxicity in an assay was calculated from the mean of quadruplicate cultures using the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{(c.p.m. with effector cells} - \text{c.p.m. spontaneous release)}}{\text{(c.p.m. total release} - \text{c.p.m. spontaneous release)}}.$$

Flow cytometry

PBML (50 μl) were incubated with monoclonal antibodies specific for bovine leucocyte surface antigens (VMRD, Inc., Pullman, WA): CD2 (B26A4), CD4 (CACT83B), CD6 (BAQ82A), CD8 (BAQ111A), WC1 (B7A), B cells (BAQ44A), MHC class II (non-polymorphic determinant, TH14B), and monocyte/macrophage/granulocyte (DH59B).[16] After washing, cells were incubated with fluorescein isothiocyanate conjugated goat anti-mouse Ig (Becton Dickinson, Mountain View, CA), washed, fixed in 2% formaldehyde, and stored at 4° until analysed. Flow cytometric analysis was done with a Coulter Electronics Ltd EPICS CS system flow cytometer. Data from 10,000 cells were collected and two-parameter analysis of forward angle versus 90° light scatter was used to gate the population of cells for fluorescence analysis. The percentage of positive cells was determined, and the percentage of cells stained using an irrelevant monoclonal antibody was subtracted to obtain the net percentage positive cells.

RESULTS

Cytotoxic activities of effector cell populations

The effect of BHV-1 infection on the ability of PBML, BLNC and LPL to mediate cytotoxicity was assessed using cells isolated from calves at different times after infection. PBML obtained from calves at 8 days post-BHV-1-infection, were

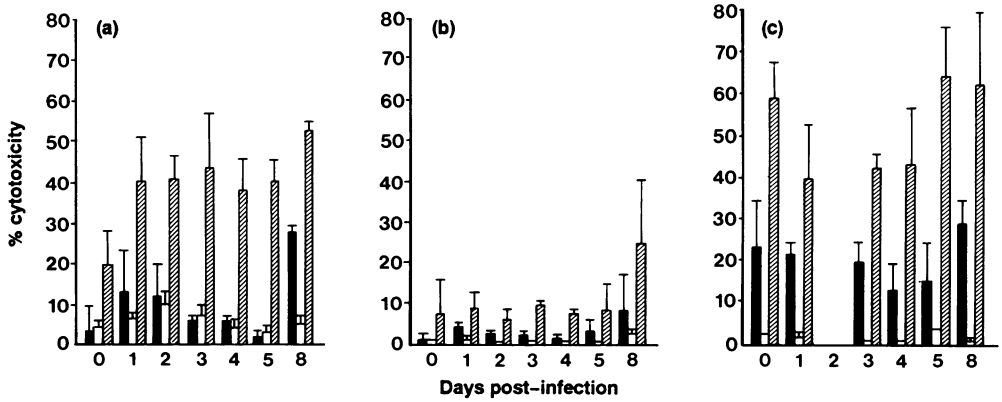

Figure 1. Effect of BHV-1 infection on the cytotoxic potential of different effector cell populations. Cytotoxicity was evaluated using peripheral blood mononuclear leucocytes (■), cranial tracheobronchial lymph node cells (□) and lung parenchyma leucocytes (▨) obtained at different times after intranasal challenge of calves with BHV-1. Cell-mediated cytotoxicity was assessed against BHV-infected (a) and non-infected GBK cells (b) and against K562 cells (c) in the presence of IL-2 using a ^{51}Cr release assay. Column height and vertical bars represent the mean of three calves ± SEM.

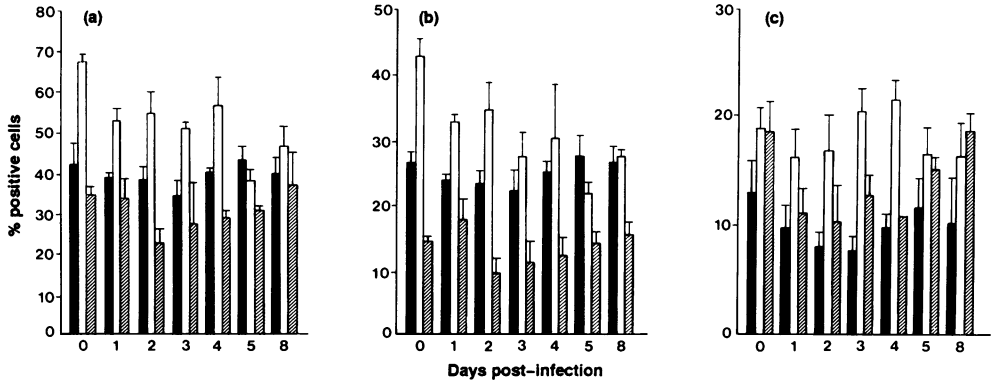

Figure 2. Phenotypic profile of effector cell populations from peripheral blood (■) cranial tracheobronchial lymph node (□), and lung parenchyma (▨) obtained at different times after intranasal challenge of calves with BHV-1. The percentage (%) of positive cells was determined by flow cytometry using monoclonal antibodies specific for bovine cell surface determinants equivalent to CD6 (a), CD4 (b) and CD8 (c). Column height and vertical bars represent the mean of three calves ± SEM.

capable of mediating cytotoxicity of BHV-1-infected GBK cells, whereas PBML from uninfected calves or from calves at other times after infection were unable to mediate similar activity (Fig. 1a). In contrast, LPL from non-infected animals caused significant lysis of BHV-1-infected cells (19·9% ± 7·7). This activity doubled (40% ± 11·5) 1 day after challenge and remained elevated at all other times tested (Fig. 1a). The cytotoxic activity against BHV-1-infected target cells mediated by BLNC from infected and non-infected calves was always below 10% (Fig. 1a). Cytotoxicity against non-infected GBK cells mediated by all effector cells was always lower than cytotoxicity against BHV-1-infected cells and only LPL obtained 8 days after challenge mediated more than 10% lysis when non-infected cells were used as target cells (Fig. 1b).

In the absence of IL-2, all effector cells mediated less than 5% cytotoxicity against K562 target cells and the level of cytotoxicity remained low after BHV-1 infection (data not shown). However, the inclusion of IL-2 (100 U/ml) in the cytotoxic assay caused a dramatic increase in the ability of LPL to mediate lysis of K562 target cells (Fig. 1c). IL-2 treatment also enhanced cytotoxicity mediated by PBML but did not augment killing by BLNC (Fig. 1c). LPL obtained 1, 3 and 4 days post-infection mediated less LAK activity than LPL from non-infected animals, whereas LPL obtained on Days 5 and 8 post-infection had cytotoxic levels similar to those of uninfected animals. A slight decrease of LAK cell activity was also observed PBML obtained 4 and 5 days post-infection (Fig. 1c).

Phenotypic analysis of effector cell populations

BHV-1 infection has been shown to affect the circulation patterns of leucocytes in the body, which could alter the proportion of different cell types in the recovered cell populations.[17] Therefore, we examined the phenotype of the effector cells used in the cytotoxicity assays by flow cytometry. PBML and BLNC populations had a larger proportion of $CD4^+$ T cells than LPL (CD4:CD8 ratio of LPL obtained from non-infected calves was 0·76) (Fig. 2b and c). The proportion of $CD6^+$ T cells was slightly reduced in all cell populations after BHV-1 infection (Fig. 2a). In peripheral blood, BHV-1 infection caused a reduction in $CD8^+$ T cells, without altering the proportion of $CD4^+$ T cells. A similar phenomenon was observed for $CD8^+$ cells in LPL population. However, the opposite trend was

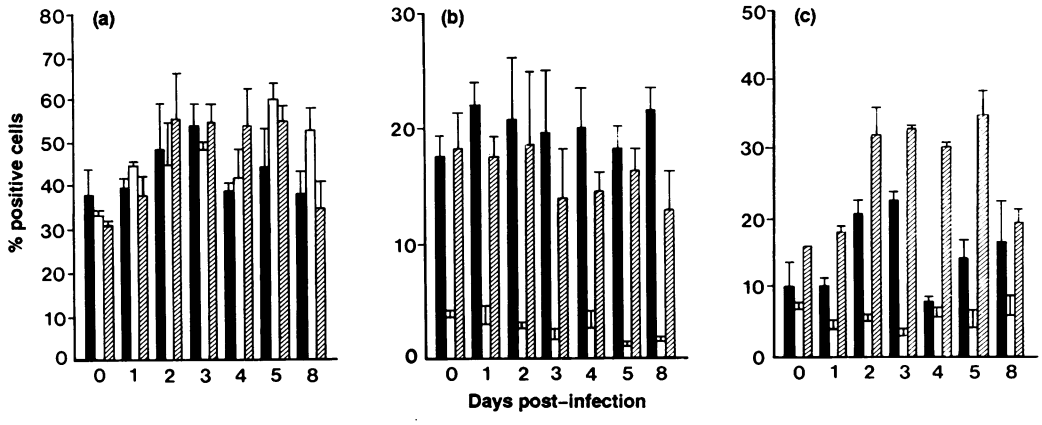

Figure 3. Phenotypic profile of effector cell population from peripheral blood (■), cranial tracheobronchial lymph node (□) and lung parenchyma (▨) obtained at different times after intranasal challenge of calves with BHV-1. The percentage (%) of positive cells was determined by flow cytometry using monoclonal antibodies specific for MHC class II antigens (TH14B) (a), WC1$^+$ cells (B7A) (b) and monocyte/macrophages (DH59B) (c). Column height and vertical bars represent the mean of three calves ± SEM.

observed in BLNC where the proportion of C4$^+$ cells was consistently lower in infected animals while the percentage of CD8$^+$ cells remained similar to that of non-infected controls (Fig. 2b and c).

After BHV-1 infection, the proportion of cells expressing MHC class II molecules increased in all cell populations (Fig. 3a). The increase was detected as early as 1 day post-infection in LPL and BLNC and by Day 2 in PBML. This increase in MHC class II$^+$ cells correlated with an increase in DH59B$^+$ (monocyte/macrophages) in PBML and LPL (Fig. 2c). In contrast, the number of monocytes in BLNC did not change after infection, and the increase in the proportion of MHC class II$^+$ cell in this population was due to an increase in the number of B cells (data not shown). Other than a slight increase in the percentage of WC1$^+$ cells in PBML after infection, the proportion of WC1$^+$ did not follow any particular trend (Fig. 3b).

DISCUSSION

The objectives of this study were to investigate whether leucocytes present in the bovine lung were able to mediate cytotoxicity and to determine the effect of BHV-1 infection on this function. The results demonstrate that leucocyte populations recovered from lung parenchyma of naive calves were able to mediate cytotoxicity against virus-infected cells and tumour cells. In addition, BHV-1 infection enhanced the cytotoxic activity of this cell population against virus-infected cells. Thus, a potentially important antiviral activity was mediated by cells present at the site of infection and was augmented in response to infection.

Two important features regarding the cytotoxic activity against BHV-1-infected cells need to be highlighted. First, the effector cells used in these experiments were obtained from an outbred population and it is unlikely that all animals shared histocompatibility antigens with the permanent cell line used as target cells. Secondly, enhanced cytotoxicity against virus-infected cells was detected as early as 1 day post-infection in LPL. These observations suggest that killing of BHV-1-infected GBK cells by LPL was mediated through a non-MHC restricted mechanism that involved rapid activation or recruitment rather than clonal expansion.

Macrophages and NK cells are the predominant effector cells of non-MHC restricted cytotoxicity and both cell types mediate lysis of BHV-1 infected cells in cattle.[4,8] Non-immune bovine PBML were suggested to be similar to the NK cell described in other species. However, the type of target cell has been influential in the detection of cytotoxicity against BHV-1-infected cells.[18] BHV-1-infected xenogeneic tumour cells (line A549)[8] and BHV-1-infected primary bovine embryonic kidney cells[16] are sensitive to lysis by non-stimulated lymphocytes, whereas BHV-1-infected GBK (the target cell used in this experiment) can only be killed by non-immune PBML after activation of the effector population with IL-2 (M. Campos, P. Griebel, H. Bielefeldt Ohmann and L. A. Babiuk, unpublished data). Similarly, lysis of K562 cells by bovine PBML is only demonstrated after IL-2 treatment.[7,8] Thus, spontaneous NK-like activity by bovine PBML is rarely detected, but IL-2 treatment of the effector cells can induce an activated NK or LAK type of activity.

In this study, LPL from non-infected calves mediated cytotoxicity against BHV-1-infected GBK cells in the absence of IL-2 activation. A similar activity was not present in PBML. While neither cell population was able to mediate lysis of K562 target cells in the absence of IL-2, LPL mediated higher levels of cytotoxicity than PBML after IL-2 activation. This observation suggests that effector cells capable of killing virus-infected cells and of responding to IL-2 with enhanced cytotoxicity are either present in greater numbers in lung parenchyma or more efficient killer cells than those found in blood. The differences in killing ability between cells from different anatomic compartments demonstrate that peripheral blood cells do not accurately reflect the functional activities of lung parenchyma cells.

The existence of NK cell activity in lung tissue has been established in other species.[13] It would appear possible that the lung cell responsible for the cytotoxicity observed after BHV-1 infection corresponds to activated NK cells. The ability of lung-derived NK cells to respond to viral infections with enhanced cytotoxicity has been described in the murine influenza virus model.[13] In that study, NK cell activity of LPL was elevated 2 days after infection with influenza virus, whereas enhancement

of NK activity in spleen cells was not detected until 4 days following challenge. The authors suggested that enhancement of lung NK activity was due to the local production of interferon or other NK-augmenting factors in response to the viral infection. They postulated that the delayed activation of splenic NK cells was due to the need for the accumulation of these factors to reach sufficient blood levels that could be 'sensed' in the spleen.[13]

We have shown that the cytotoxic activity of LPL against tumour cells was reduced early after BHV-1 infection (Days 1 and 3 post-infection). In contrast, cytotoxicity of LPL against BHV-1-infected cells was consistently higher in infected animals, suggesting that killing of K562 target cells and of BHV-1 infected cells is likely mediated by different effector cell mechanisms. The heterogeneity of non-MHC restricted cytotoxic mechanisms has been well documented and it has been suggested that killing of tumour target cells and virus-infected cells by NK cells is mediated by different mechanisms.[19] Unlike tumour cell lysis, killing of virus-infected cells has been shown to require activation signals delivered by accessory cells.[20-22] It is possible that BHV-1 infection could induce the recruitment and/or activation of such accessory cells, resulting in more effective lysis of virus-infected cells.

Previously, the most prominent cytotoxic function observed after infection of cattle with BHV-1 has been a non-MHC restricted mechanism mediated by PBML that can be detected using BHV-1-infected GBK cells at approximately 1 week after BHV-1 challenge.[4,9] Killing of the BHV-1-infected cells was mediated by a cell belonging to the monocyte lineage and it was proposed that this activity was dependent upon activation signals delivered by 'immune' lymphocytes.[4] Later studies demonstrated that IFN-γ was required but not sufficient for the induction of this activity.[10] In the present study only PBML obtained from calves 8 days after BHV-1 challenge were able to mediate lysis of BHV-1-infected GBK cells. Phenotypic analysis of the effector cell populations showed that cells obtained from the lungs of infected calves had an increased proportion of monocyte/macrophages and a corresponding increase in the number of cells expressing MHC class II molecules. The phenotypic changes suggest that IFN-γ production and macrophage activation are predominant features of BHV-1 infection and it is possible that the enhancement in cytotoxicity of BHV-1-infected cells by LPL is mediated through a mechanism similar to that described in PBML.

In summary, we describe a non-MHC cytotoxic mechanism against virus-infected cells which was enhanced in LPL shortly after virus challenge (1 day) suggesting that the cells responsible for this activity are present in the lung parenchyma and that BHV-1 infection either induces a rapid activation of the existing cytotoxic populations and/or promotes the additional recruitment of cells able to perform this function. However, without further characterization of this cell population, it is unknown whether the enhanced cytotoxicity observed in LPL from both BHV-1-infected and non-infected animals is mediated by the same cell or whether BHV-1 infection influences the recruitment and/or activation of another cytotoxic cell type.

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