### Introduction

Bovine herpesvirus-1 (BHV-1) is a major pathogen in cattle, and is the causative agent of a variety of diseases including infectious bovine rhinotracheitis, conjunctivitis, enteritis, encephalomyelitis and infectious pustular vulvovaginitis (Denis et al., 1994). Common secondary bacterial infections may result in bronchopneumonia and death. However, the interaction of virus with immune cells that contribute to initial viral infection has never been precisely defined.

Depressed cell mediated immune responses have been observed with herpesvirus infections. Previous studies have shown that IL-2 production (Babiuk & Bielefeldt Ohmann, 1985), responses to mitogen (Babiuk & Bielefeldt Ohmann, 1985; Bielefeldt Ohmann & Babiuk, 1985b) and natural cytotoxicity responses (Babiuk & Bielefeldt Ohmann, 1985; Bielefeldt Ohmann & Babiuk, 1985a) are lower in BHV-1 infected animals. Several theories have been proposed to explain herpesvirus induced immunosuppression. T cells may provide an inadequate level of help (Babiuk & Bielefeldt Ohmann, 1985). Alternatively, suppression of immune responses of peripheral blood mononuclear cells by BHV-1 may be due to a selective depletion of certain lymphocyte populations or by changes in lymphocyte trafficking (Filion et al., 1981; Griebel et al., 1987, 1988). Recent studies have shown that proteins produced by herpesviruses interfere with class I mediated antigen presentation. Specifically, the herpes simplex virus (HSV) protein ICP47 blocks the formation of class I–antigen complexes by preventing transport of peptides into the endoplasmic reticulum and thus preventing the generation of virus specific cytotoxic T lymphocytes (Fruh et al., 1995). To date, no bovine ICP47 homologue has been identified. An early protein produced during Epstein–Barr virus (EBV) infection also interferes with class I mediated presentation (Levitskaya et al., 1995), but the mechanism is currently unknown. Studies of HSV and EBV suggest that peptide association with MHC class I is depressed, resulting in decreased CD8+ T cell function. However, the ability of BHV-1 to affect T lymphocytes is unknown.

Depletion of specific cell populations or loss of cell surface molecules necessary for antigen recognition during infection have important biological ramifications. To control viral infections, a variety of humoral, cytokine and cellular responses are produced by the host in an orchestrated fashion. As herpesviruses spread intracellularly and are capable of existing...
in the presence of virus specific antibodies, cell mediated immunity is believed to be more important than humoral immunity in the elimination of virus (Collins et al., 1985). Following engagement of the CD4 molecule with peptide and MHC class II on antigen-presenting cells, CD4+ T cells produce cytokines required by CD8+ cytotoxic T cells as well as natural killer cells, macrophages, B cells and proliferating T cells. Therefore, selective depletion of CD4+ T cells or absence of the CD4 molecule directly affects CD4+ T cell function and cytokine signalling to antibody-producing B cells, as well as cytotoxic T cells participating in the response to BHV-1.

Several viruses are selectively tropic for certain cell populations. Human immunodeficiency virus (HIV) specifically lyses CD4+ cells (Groux et al., 1992). Vesicular stomatitis virus lyses activated T cells (Hom et al., 1989), as does HSV (Pelton et al., 1977). Human herpesvirus-6 infects CD4+ T cells and a small percentage of CD8+ cells (Takahashi et al., 1989).

Early studies indicate that BHV-1 infects monocytes (Rossi & Kiesel, 1977). Previously, we have shown that BHV-1 can bind monocytes and lectin-activated lymphocytes (Splitter & Eskra, 1986). Attempts to stimulate activated T cells (Griebel et al., 1990) with live BHV-1 have resulted in cell death. Although inhibition of proliferative responses and cell death have been observed with BHV-1 infected lymphoblasts, isolation of infectious virus has failed (Carter et al., 1989; Griebel et al., 1990). Furthermore, using glycoprotein-specific monoclonal antibodies, viral proteins could not be detected on metabolically labelled infected ConA activated cells using immunoprecipitated cell lysates and PAGE (Griebel et al., 1990). However, this methodology lacks sensitivity in detecting low levels of protein. Also, the population(s) of cells affected by virus remained undetermined.

Herpesviruses use a variety of mechanisms to elude the host’s immune system. By spreading intracellularly, herpesviruses can exist in the presence of virus specific antibodies. Cytotoxic T cell activity is prevented by virus interference in class I mediated antigen presentation. In this study, we demonstrate that BHV-1 glycoproteins are found on CD4+ T cells infected in vitro and that immediate early/early gene translation is initiated in activated and resting lymphocytes. However, CD4+ cells in the presence of live BHV-1 lose CD4 molecule expression and apoptosis occurs. These findings indicate that immune suppression during BHV-1 infection may also result from the selective depletion of CD4+ lymphocytes and thus provide the virus with an advantage in the initial stages of infection.

### Methods

**Virus.** Cooper strain (ATCC VR-864) of BHV-1 was harvested from MDBK cells 72 h after infection. Supernatant was collected, centrifuged at 9500 g for 10 min, aliquotted and frozen at −70 °C. Virus titre was determined by plaque assay using MDBK cells (ATCC CCL 22).

**Animals.** Animals were housed at the University of Wisconsin Dairy Cattle Center.

**Cell culture.** Peripheral blood mononuclear (PBMC) cells were obtained by density gradient centrifugation. Cells were cultured at 2 × 10^6/ml in RPMI 1640 supplemented with 10% foetal bovine serum, 2 mM l-glutamine, 2 × 10^−5 2-mercaptoethanol, 100 µg/ml streptomycin and 100 U/ml penicillin. ConA was added at 2.5 µg/ml and BHV-1 was added at the concentration and time indicated in the text. For some experiments, BHV-1 was UV irradiated for 7 min at a distance of 11 cm from a UV lamp. Plaque assays were performed to determine that UV-irradiated virus was inactivated.

**Monoclonal antibodies.** Monoclonal antibodies (MAbs) to three glycoproteins of BHV-1 (provided by G. Letchworth, University of Wisconsin) were used. Antibodies 480704, 150704 and 110604 recognized glycoproteins B, C and D, respectively (Ludwig & Letchworth, 1987; Marshall et al., 1986). MAbs to bovine CD4 (IL-12A) and γ/δ (IL-A29) were produced by the International Laboratory for Research on Animal Diseases (Baldwin et al., 1986). MAbs 38-65 and 19-19 to ovine SBU-T8 and γ/δ (Mackay et al., 1986), cross-reactive with bovine CD8 and bovine γ/δ (O’Reilly & Splitter, 1989), respectively, were purchased from the University of Melbourne (Parkville, Victoria, Australia). MAb 33, an anti-bovine IgM, was a gift from Klaus Nielsen (Animal Research Institute, Ontario, Canada). P1.17 (ATCC TIB10) and P3X63Ag8 (ATCC TIB9) cell lines were purchased from the ATCC to produce ascites for use as isotype controls. C5B6 MAb, specific for bovine CD11c, was used to identify monocytes (Eskra et al., 1991). MAbs were biotinylated with sulfo-NHS-biotin according to the manufacturer’s recommendations (Pierce).

**Immunofluorescence.** One- and two-colour immunofluorescence was performed as described previously (O’Reilly & Splitter, 1990). Fluorescein conjugated goat anti-mouse (GAM) IgG (H+L) and B-phycoerythrin-conjugated streptavidin (PE streptavidin) were obtained from Jackson Immunoresearch Laboratories. conjugates were used at optimal saturating concentrations (data not shown). A Coulter Electronics Epics flow cytometer was used to determine the percentage of fluorescent cells, and a Becton Dickinson FACS Star Plus was used for cell sorting.

**Detection of apoptosis.** PBMC cells were cultured with ConA for 4 days. BHV-1, at an m.o.i. of 10, was added for the time indicated prior to harvesting. Background apoptotic levels were determined using uninfected cells. To detect apoptosis at the single cell level, an in situ cell detection kit (Boehringer-Mannheim) was used according to the manufacturer’s directions. Briefly, cells were fixed with paraformaldehyde and permeabilized with Triton X-100. Terminal deoxynucleotidyl transferase-mediated fluorescein conjugated-dUTP nick end labelling (TUNEL) was used to label DNA strand breaks. For two-colour microfluorimetry, antibody to cell surface markers was added followed by PE streptavidin. Cells were then fixed, permeabilized and labelled as described above for detection of apoptosis.

Apopotic death was verified by ELISA (Boehringer-Mannheim). Cytoplasmic fractions of cell culture were incubated in microtitre wells coated with anti-histone MAb. Anti-DNA–peroxidase was added, followed by the substrate 2,2-azino-di-[3-ethylbenzthiazoline sulfonate]. Cytoplasmic fractions were diluted 1:50 prior to use.

**RNA isolation and reverse transcription.** RNA was isolated using guanidium thiocyanate (TRI) (Molecular Research Center) according to the manufacturer’s directions. RNA was treated with 4 units of RNase-free DNase in 40 mM Tris–HCl, pH 8.0, 10 mM NaCl, 6 mM MgCl₂ and 10 mM CaCl₂ for 1 h at 37 °C and then at 60 °C for 10 min. Reverse transcription (RT) reactions were performed in the presence or absence of Moloney murine leukaemia virus (M-MLV) reverse transcriptase at 42 °C using a reverse transcription system (Promega).
Primers and PCR. Primers used for amplification of immediate-early/early gene (IER2.9/ER2.6) (Wirth et al., 1992) by PCR were designed using Oligo 4.0 (National Biosciences, Plymouth, Minn., USA). IE-539 (CGACCTGACCAGCACCTTGAGACG) and IE-849 (ACGGGCTCTCCCTCCTTCTCTCTG) were designed based on sequence data for BHV-1 (accession number M84464). Primers GPD-13 (ACATTGGCCGTGCGCGGCGG) and GPD-305 (AAACGGGGTGTGGCGGCGG) were used for gD amplification (Xiaodi Ren, University of Wisconsin). Amplification of bovine IL-1α was performed as previously described (Covert & Splitter, 1995). PCR amplification was carried out in 50 μl consisting of 200 μM deoxyribonucleoside triphosphate (dNTP), 10× buffer, 1.25 Units Taq polymerase, 0.2 μM primers and 4% dimethylsulphoxide. PCR was performed for 35 cycles (1 min at 95 °C, 1 min at 55 °C, 2 min at 72 °C) in a thermocycler. After a final extension for 5 min at 72 °C, PCR products were analysed on a 1.5% agarose gel containing ethidium bromide.

Results

CD4 T lymphocytes express viral glycoproteins

Lymphocytes stimulated with ConA for 3 days were cultured for the final 24 h with BHV-1 and stained with MAb to cell surface receptors and viral glycoproteins. Fig. 1 shows a typical two-colour profile. More than 40% of the CD4+ T cells expressed viral glycoproteins. The number of CD4+ T cells expressing viral glycoproteins ranged from 27–49% with an average of 38% (n = 5 experiments). Alternatively, to confirm that CD4+ T lymphocytes were expressing viral glycoproteins, lymphocytes were first isolated by fluorescent activated cell sorting using a MAb specific for the CD4 molecule, followed by reanalysis of the isolated cells for viral glycoprotein expression. Of the CD4+ T cells isolated by fluorescent activated cell sorting, 50% expressed viral glycoproteins (data not shown).

In contrast, activated CD8+ T cells expressing viral proteins ranged from 5–17% (average 11%, n = 5 experiments). The percentage of cells expressing glycoproteins was always lower in the CD8+ than in the CD4+ T cell population (Fig. 1). Because low numbers of CD8+ T cells were present, viral glycoprotein expression on CD8+ T cells by fluorescent activated cell sorting was inconclusive.

To determine if cell phenotypes other than CD4 and CD8 were infected by virus, antibodies to other cell surface markers were used. Antibodies to B cells (anti-IgM) and monocytes (C5B6) revealed that very few of these cells (< 3%) were present in the nonadherent cell population. Variable numbers of γ/δ lymphocytes (identified by SBU-T19 and IL-A29) were present in ConA activated cells, but minimal expression (< 3%) of viral glycoproteins was observed on γ/δ lymphocytes (data not shown).

BHV-1 lyses ConA activated cells

At low concentrations of virus, few cells expressed viral glycoproteins (data not shown). Glycoprotein expression was not detected at 5 h post-infection, but expression increased from 24 to 48 h (data not shown). Correspondingly, a decrease in the number of cells expressing the CD4 receptor was observed (Table 1). Decreased detection of CD4 expression was observed in all three animals tested. However, the number of CD8+ T cells remained relatively constant or increased during the 48 h experimental period. A decrease in cells expressing the CD4 receptor could be due to loss of the CD4 molecule or to lysis of CD4+ T cells. As a decrease in the number of cells expressing the CD2 molecule was also observed, viability studies were conducted to determine if BHV-1 caused lysis of ConA activated cells. Increased cell death was observed at 48 h, paralleling the decrease of CD4+ T cells. By 48 h post-infection, viability of virus infected cells was reduced to 29% compared to 83% for uninfected cells, supporting the hypothesis that BHV-1 contributes to lysis of CD4+ T cells. Increased cell death was also observed with...
Table 1. Percentage of CD4 and CD8 T cells is altered during BHV-1 infection

<table>
<thead>
<tr>
<th>Cell phenotype*</th>
<th>24 h</th>
<th>48 h</th>
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<tr>
<td>−BHV-1 + BHV-1</td>
<td>−BHV-1 + BHV-1</td>
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<td>CD4</td>
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<td>32</td>
<td>18</td>
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<td>38 ± 5</td>
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<td>Mean ± SD</td>
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<td>13 ± 3</td>
<td>15 ± 2</td>
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* Percentage staining determined by flow cytometry using MAbs specific for CD4 (IL-A12) or CD8 (38-65) in three separate experiments.

Fig. 2. Apoptosis detected by flow cytometry. ConA activated cells were harvested 72 h after the initiation of culture. Aliquots of cells were infected with BHV-1 (m.o.i. = 10) 22, 24, 28, 32 or 44 h prior to harvesting. Mock infected ConA activated cells (cultured for 72 h without BHV-1 and represented by 0 h post-infection) were used as a control to detect background levels of apoptosis. Means ± standard deviations are shown. Absorbance was measured at 405 nm.

Cell lysis of virus-infected Con A activated cells is due to apoptosis

To determine if the cell death observed in virus infected cultures was due to apoptosis, two apoptosis detection assays were performed. No increase in apoptosis was detected by flow cytometry with cells infected for 7 h but increased fluorescence was observed at later times (Fig. 2) with up to 83% of cells fluorescing. As fluorescence detected at late time points may be due to necrosis, anti-histone MAb based ELISA assays were performed to verify apoptosis. As seen in Fig. 3, apoptotic death was observed at 24 h with two of the animals tested and by 48 h with all of the animals. This apoptotic cell death paralleled detection of viral glycoprotein expression and cell viability data.

Two-colour immunofluorescence was performed to determine if apoptotic cells were of the CD4+ phenotype (Fig. 4). Twenty-four percent of uninfected cells underwent apoptosis, of which 16% was contributed by the CD4 fraction and 8% by the CD8 fraction. Forty-eight h after the addition of BHV-1, apoptosis increased to 51%, of which 29 and 9%, respectively, possessed the CD4 and CD8 molecules. The remaining 13% were CD4+CD8− cells. A 12% decrease in CD4+ cells was observed 48 h after infection, paralleling the increase in apoptotic CD4+CD8− cells. The number of cells expressing the CD8 receptor remained unchanged, as did the percentage of CD8+ cells undergoing apoptosis. Thus an increase in CD4+ cells undergoing apoptosis was observed 24 and 48 h after the addition of BHV-1, despite a decrease in the number of cells expressing the CD4 molecule. Analysis of the CD4+ cells revealed that 23, 39 and 49% were apoptotic at 0, 24 and 48 h post-infection, respectively (data not shown). These results are analogous to our earlier experiments (Table 1) where a decrease in CD4+ but not CD8+ molecules was observed. Furthermore, as seen previously, not all CD4+ cells were affected.

To verify that CD4+ cells undergo apoptosis in the presence of BHV-1, CD4+ cells were isolated using flow cytometry. BHV-1 was added to ConA activated cells 24 h before labelling with anti-CD4 MAb and secondary antibody. CD4 cells were isolated and ELISA assays performed. An increase in apoptosis of CD4+ cells in the presence of BHV-1 was observed (data not shown).
Live virus is required for glycoprotein expression

Because proliferative responses to ConA are blocked by live but not by UV irradiated BHV-1 (Griebel et al., 1990), we then tested whether viral glycoproteins from killed virus could be detected on the surface of CD4+ cells. UV irradiated virus was added to ConA activated cells for 24 or 48 h followed by flow cytometry. Table 2 is representative of three experiments. Glycoproteins could not be detected on the surface of lymphocytes following addition of UV treated virus compared to live virus in all three experiments.

Viral gene expression occurs in ConA activated cells

PCR was done to determine if specific virus replication stages occurred in ConA cells. RNA was isolated from day 3 ConA activated cells 5 h after virus infection. Reverse transcription was performed following DNase treatment to reduce the possibility of contaminating viral DNA. Reactions without M-MLV reverse transcriptase ensured that the PCR product was not due to contaminating viral DNA. As seen in Fig. 5(A), transcription of the immediate early/early IER2.9/IER2.6 genes occurred in virus infected ConA activated cells similar to virus infected MDBK cells. IER2.9/IER2.6 genes were also detected in cells cultured without ConA (data not shown).

BHV-1 replication proceeds in a cascading manner with immediate-early gene replication followed by early and then late gene replication. Primers to an early gene (gD) were used to assess subsequent viral gene transcription. Early gD gene products could also be detected in virus infected ConA activated cells (Fig. 5B).

To verify that replication of BHV-1 occurred in CD4+ cells, subpopulations of T cells were isolated by flow cytometry. BHV-1 infected day 3 ConA activated cells were labelled with monoclonal antibodies to the CD4 or CD8 molecule. Following flow cytometry, RNA was isolated from the CD4+ and CD8+ populations and RT–PCR was performed. ConA activated mock and BHV-1 infected cells were used as controls. Transcription of the IER2.9/IER2.6 gene occurred in virus infected CD4+ but not CD8+ cells (Fig. 6).

BHV-1 has been shown to replicate in monocytes (Rossi & Kiesel, 1977) and these cells may function to disseminate virus in the host. Flow cytometry data showed monocyte contamination of ConA activated cells to be less than 3%. As bovine macrophages constitutively produce IL-1α (Covert & Splittler, 1995), primers to IL-1α were used to determine if viral gene transcription from contaminating monocytes might occur. No IL-1α product could be detected by PCR using day 3 ConA activated cell populations, supporting the hypothesis that viral gene transcription was detected from lymphocytes and not monocytes (data not shown).

Discussion

Depressed cell mediated immune responses in cattle infected with BHV-1 have been well documented (Babiuk & Bielefeldt Ohmann, 1985; Bielefeldt Ohmann & Babiuk, 1985a; Filion et al., 1981). Workers have demonstrated that inhibition of mitogenic responses in cells infected with BHV-1 is due to cell death (Griebel et al., 1990). In contrast, others have reported that inhibition of proliferative responses is not caused by cell death, as cytotoxic functions remain intact (Carter et al., 1989). T cell death has been observed with other virus infections: HIV (Groux et al., 1992; Ho et al., 1987), EBV (Uehara et al., 1992) and lymphocytic choriomeningitis virus (Razvi & Welsh, 1993).
This discrepancy has prompted us to examine in greater detail the effect of the virus on lymphocytes. Our data indicate that cell lysis is occurring and the predominant cell type affected is CD4+ T lymphocytes. However, not all CD4+ cells express virus glycoproteins (Fig. 1), nor do all of these cells undergo apoptosis (Fig. 4). This result could explain the maintenance of lectin dependent cytotoxic abilities by non CD4+ T lymphocytes observed by others (Carter et al., 1989), while proliferative responses are inhibited. Furthermore, as not all CD4+ T cells are infected, it is possible that selected helper T cell subpopulations persist and thus certain CD4+ T cell functions remain unchanged.

Previously, Carter et al. (1989) have shown that less than one cell in 1000 ConA activated lymphocytes is productively infected with BHV-1. These authors postulated that suppression of proliferative responses could result from either a nonpermissive or a nonproductive infection. Similarly, live but not UV irradiated virus suppressed proliferation. However, viral proteins were not detected in metabolically labelled, infected lymphoblasts by PAGE, nor were early nuclear particle formation, capsid assembly or envelopment observed by electron microscopy (Griebel et al., 1990). Attempts to isolate infectious virus from infected lymphoblasts have been unsuccessful. These findings parallel results seen with cytomegalovirus (CMV) (Rice et al., 1984). Lymphocyte proliferation to lectin and natural killer cell lytic activity are inhibited in lymphocytes infected with CMV. However, infectious virus was not produced nor could mature virions be visualized. Interestingly, CMV infects lymphocytes and monocytes but only the immediate-early polypeptides are synthesized. Unlike studies using BHV-1, the viability of the cells was not affected by virus infection. In contrast to earlier work with BHV-1 (Griebel et al., 1990), we determined that an immediate early/early (IER2.9/ER2.6) and early (gD) genes can be detected using RT–PCR. The possibility of contaminating monocytes contributing to the detection of immediate-early genes cannot be completely eliminated. However, the simultaneous detection of CD11c+ (a molecule present on monocytes) and BHV-1 glycoproteins was not evident. Furthermore, IL-1α production, as an additional indication of monocyte presence, was not detected in either virus or mock infected cultured cells. If constitutive IL-1α production was not detected, then immediate-early and early gene transcription from these cells would probably not be measurable. Isolation of RNA from subpopulations of T cells revealed that IER2.9/ER2.6 gene products are produced in CD4+ but not CD8+ cells. As production of mature virions occurs in a cascading fashion, incomplete protein synthesis most probably occurs in CD4+ T cells, leading to cell lysis and production of incomplete virions. Although immediate-early/early gene products are produced, the extent of additional viral gene
transcription and viral gene(s) responsible for cell death has not been determined. However, using flow cytometry, early viral glycoproteins (gB and gD) were detected on the cell surface while late (gC) glycoproteins were not (data not shown).

Using immunofluorescence, we have found viral glycoproteins on the cell surface of ConA activated CD4+ cells. These glycoproteins could be detected on increasing numbers of cells even when the virus had been removed by washing prior to detection of cell surface viral glycoproteins. The number of CD4+ T cells expressing viral glycoproteins decreased 48 h after the addition of virus, while the number of CD8+ T cells remained constant and in some instances increased. Furthermore, our findings indicate that detection of viral glycoproteins on ConA activated cells requires infectious BHV-1.

Characteristic cell changes indicative of apoptosis have been observed in BHV-1 infected cells. The requirement for replicating virus to initiate apoptosis has been described using HSV (Tropea et al., 1995). We have determined that live virus is required for cell surface glycoprotein expression as well as inhibition of proliferative responses. Our findings of apoptosis in a subpopulation of CD4 T cells extend the preliminary observations of others (Griebel et al., 1990) reporting nuclear disintegration of lymphoblasts. Following early HSV gene production in lymphoid cells, chromatin condensation and disaggregation of the nucleoli occurs (Roizman & Sears, 1990), suggesting a pattern of cell death in alphaherpesvirus infection.

Activation of peripheral blood and lymph node lymphocytes and lymphocyte infiltration into the submucosa of the respiratory tract is a common immune occurrence during BHV-1 infection (Allan & Mosolla, 1980). Our findings indicate that, central to this cellular immune response, ConA activated CD4+ T cells can be infected by BHV-1. Although infectious virus may not be produced, lysis of CD4+ T lymphocytes would have important implications for the course of virus infection in the respiratory tract and regional lymph nodes. The interaction of virus and activated lymphocytes in vivo remains to be determined, but our data strongly support the ability of BHV-1 to selectively infect CD4+ T cells, rapidly reduce CD4 expression, and lyse these cells. The concept of virus infection of significant numbers of CD4 T lymphocytes provides a rationale for the poorly understood observation of early cell mediated immunosuppression. Our findings now provide a mechanistic basis for the initial preference of virus in the host compromised in helper lymphocyte function.

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References


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