The interaction between bovine herpesvirus type 1 and activated bovine T lymphocytes

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The interaction between activated bovine T lymphocytes (BTLs) and bovine herpesvirus type 1 (BHV-1) was investigated. BHV-1 infection of BTLs reduced the amplitude of recombinant bovine interleukin 2-induced proliferative responses. This decreased proliferation was caused by a virus-induced lymphocytolysis which was dependent on viable virus and was not inhibited by recombinant bovine interferon-α-1. Furthermore, lymphocytolysis was not associated with virus replication or with the synthesis of detectable levels of viral proteins. Electron microscopic examination of virus-infected cells revealed that lymphocytolysis was characterized by early nuclear disintegration resembling apoptosis. These observations suggest that activated T cells, localized at the site of BHV-1 infection, may be susceptible to virus-induced cytolysis.

Introduction

Bovine herpesvirus type 1 (BHV-1), a member of the Alphaherpesviridae subfamily, is a major pathogen of cattle. This virus causes a variety of diseases which include respiratory infections, conjunctivitis, encephalitis, genital infections, abortions and a fatal multisystemic infection of neonates (Gibbs & Rwemamvu, 1977; Yates, 1982). Acute BHV-1 respiratory infections, which render cattle susceptible to a fatal Gram-negative bacterial secondary pneumonia (Yates et al., 1983), are a major cause of economic losses to the North American cattle industry.

Much of the present data suggest that the immunomodulatory effects of BHV-1 infection may be an important factor contributing to bacterial superinfection (Bielefeldt Ohmann & Babiuk, 1985, 1986; Filion et al., 1983; Forman & Babiuk, 1982). In particular, BHV-1 can infect alveolar macrophages (Forman & Babiuk, 1982; Bielefeldt Ohmann et al., 1984; Forman et al., 1982) and peripheral blood monocytes (Nyaga & McKercher, 1980), and alter their functions. However, in vitro studies of the interactions between bovine T lymphocytes (BTLs) and BHV-1 indicated that peripheral blood lymphocytes bound very little radiolabelled virus (Splitter & Eskra, 1986) and that there was no viral replication in lymphocyte cultures (Nyaga & McKercher, 1980; Rouse & Babiuk, 1975). These studies suggested that a direct interaction between BHV-1 and bovine leukocytes was limited to monocytes/macrophages.

Similar studies concerning the interactions between herpes simplex virus type 1 (HSV-1) and human leukocytes indicated that monocytes/macrophages were permissive for HSV-1 replication, whereas peripheral blood lymphocytes were non-permissive (Mintz et al., 1980; Plaeger-Marshall & Smith, 1978a). However, HSV-1 could replicate in all human T lymphocyte subsets when they had been activated by antigens, mitogens or interleukin 2 (IL-2) (Plaeger-Marshall, 1978b; Hammer et al., 1982; Hammer & Gillis, 1985). Infection of T lymphocytes by HSV-1 resulted in a loss of function (Pelton et al., 1980) secondary to virus-induced lymphocytolysis (Hammer & Gillis, 1985). These data suggested that a direct interaction between HSV-1 and activated T lymphocytes could alter immune responses.

Following BHV-1 infection there is a marked lymphocyte infiltration in the submucosa of the respiratory tract (Allan & Mosolla, 1980; Allan et al., 1980; Jericho & Le Q. Darcel, 1978) and activation of peripheral blood T lymphocytes with increased IL-2 production and responsiveness (Griebel et al., 1988). These observations, together with data from HSV-1 investigations, suggest that an interaction between BHV-1 and activated BTLs
may partly account for the BHV-1-induced alteration of immune responses.

It is now possible to generate and maintain long-term cultures of BTLs (Baker & Knoblock, 1982; Carter et al., 1986; Lawman et al., 1988). These cultures provide populations of T lymphocytes which are activated and highly responsive to IL-2. A culture of IL-2-dependent BTLs was generated and maintained in our laboratory. These cells were then used to characterize the interaction between BHV-1 and activated BTLs.

**Methods**

**Virus.** BHV-1 strain 108 was originally isolated from the placenta of a cow which had aborted (Jericho & Le Q. Darcel, 1978). Stock cultures of virus were grown in monolayers of Georgia bovine kidney (GBK) cells as described previously (Rouse & Babiuk, 1975). Briefly, confluent monolayers of GBK cells were grown in 175 cm² tissue culture flasks and infected at an m.o.i of 1. When cultures displayed extensive c.p.e., they were harvested. Virus was purified as described previously (Rouse & Babiuk, 1974). This titre of infectious virus (p.f.u.) was determined in a microtitre plaque assay as described by Rouse & Babiuk (1974).

Virus was inactivated by exposure to u.v. irradiation. One ml of purified virus was placed in a 35 mm plastic Petri dish and irradiated for 3 min using two u.v. bulbs (General Electric G875) at a distance of 11 cm. The virus stock was non-infectious after this treatment.

**Cells.** Either GBK or MDBK cells were used for growing and assaying BHV-1. These cells were screened periodically to ensure the absence of *Mycoplasma* spp. common to both domestic animals and man. The screening was conducted at the Diagnostic Bacteriology Laboratory, Department of Veterinary Microbiology, Western College of Veterinary Medicine, Saskatchewan, Canada, using routine culture methods.

The cells were cultured in Eagle’s MEM supplemented with 10% foetal bovine serum (FBS), 2 mM-glutamine, 10 μl/ml of non-essential amino acids (Gibco, no. 114), 50 mg/l of gentamicin and 2.5 g/l of sodium bicarbonate.

**Cytokines.** The recombinant bovine IL-2 (rBolL-2) was a dessicated, purified preparation kindly provided by Dr P. E. Baker, with the permission of Immunex Corporation, Seattle, Wash., U.S.A. The rBolL-2 was expressed in yeast and was secreted intact but without glycosylation, and was purified to homogeneity by direct application of the crude yeast medium to reverse-phase HPLC (Baker, 1987; Price et al., 1987). Upon receipt, the activity in each vial of rBolL-2 was titrated with BTLs to determine the concentration (units/ml) that stimulated maximum lymphocyte proliferation responses (LPRs). This optimal concentration was used in subsequent assays.

The recombinant bovine interferon-γ1 (rBolIFN-γ1) (Lot No. 1942-23) was kindly supplied by Ciba-Geigy Canada. The rBolIFN-γ1 was expressed in *Escherichia coli* and purified to homogeneity as determined by HPLC and SDS-PAGE. The dessicated preparation had a specific antiviral activity of 1·8 × 10⁶ units/mg and less than 0·1 μg endotoxin per mg of protein, as determined by the Limulus amoebocyte lysate test (Whitaker Corporation).

**Flow cytometry.** The monoclonal antibodies (MAbs) used to determine the BTL phenotype were developed and characterized at the Washington State University Monoclonal Antibody Centre at Pullman, Wash., U.S.A. (Davis et al., 1987). The following MAbs were used to identify bovine leukocyte antigens: B26A (T lymphocyte–BoT2 antigen), B7A (null or N lymphocyte antigen); Pg45A (B lymphocyte–slgM) and DH59B (granulocyte/monocyte antigen). The indirect labelling procedure used has been described in detail (Griebel et al., 1988). Controls for non-specific labelling involved incubating cells with either fluorescein isothiocyanate-labelled F(ab')₂ goat anti-mouse Ig alone or in combination with an isotype-matched MAb specific for an irrelevant antigen. After labelling, all samples were fixed in a 2% solution of formaldehyde in phosphate-buffered saline (PBS) and then stored in the dark at 4 °C. All samples were analysed with a Coulter Electronics EPICS V flow cytometer.

**Bulk T cell cultures (BTCs).** The BTCs were generated from Percoll-isolated peripheral blood mononuclear cells (PBMC) cultured in 60 mm Petri dishes at a density of 1 × 10⁷ cells per ml of RPMI 1640 medium containing 5% FBS and 10 μg/ml concanavalin A (Con A). The cultures were incubated for 4 days at 37 °C, in a humidified 5% CO₂ atmosphere. The lymphoblasts were then collected, layered on Percoll (1·078 g/ml) and centrifuged at 400 g for 30 min. Cells at the interface were collected, washed three times with Hank’s balanced salt solution (HBSS), and 2 × 10⁵ cells per well were plated in six-well plates. The culture medium used was Dulbecco’s modified Eagle’s MEM (DMEM) supplemented with 5% FBS, 2 mM-glutamine, 2 × 10⁻⁵ M-2-mercaptoethanol, 1 mM-HEPES, 50 mg gentamicin/l and 15% Con A-conditioned medium (CA-CM).

The BTCs were passaged five times at 4 day intervals. With each passage the non-adherent cells were collected from the wells, washed three times with HBSS, and replated at a density of 2 × 10⁶ cells/well in new six-well plates. This procedure reduced the co-passaging of bovine monocytes. The resulting cell population proliferated in response to low doses of rBolL-2 (less than 2 units/ml) and low doses of Con A (0·5 μg/ml). The phenotype of these cells, as determined with MAbs and flow cytometry, was greater than 97%, B26A-positive (BoT2), DH59B-negative (monocyte/granulocytes), Pg45A-negative (slgM), and B7A-negative (‘null’ cells). The BTCs were passaged repeatedly for 2 months, and during this time maintained a doubling time of 18 to 24 h, with a stable phenotype. Cells from these cultures were cryopreserved in aliquots of 2 × 10⁷ cells per 1·8 ml vial. The cryopreservation medium was DMEM containing 20% FBS and 10% DMSO. When cryopreserved BTCs were recovered the cells were passaged twice with CA-CM prior to being used.

The protocol for generating CA-CM was modified from that described previously by Baker & Knoblock (1982). Briefly, bovine PBMC were cultured in 100 mm Petri dishes at a density of 1 × 10⁷ cells per ml of DMEM supplemented with 5% FBS, 2 mM-glutamine, 2 × 10⁻⁵ M-2-mercaptoethanol and 1 mM-HEPES. The cultures were incubated at 37 °C in a humidified 5% CO₂ atmosphere for 48 h and 5 μg/ml Con A was added during the final 24 h. Culture supernatant (CA-CM) was collected, centrifuged at 80 g for 7 min, and filtered through a 0·22 μm filter (Millipore) before storage of samples at −70 °C.

**Lymphocyte proliferation assays.** The proliferative responses of BTLs were measured by incubating six replicate cultures of 5 × 10⁶ cells, in a total volume of 0·2 ml DMEM, supplemented with 5% FBS, 2 mM-glutamine, 2 × 10⁻⁵ M-2-mercaptoethanol, and 1 mM-HEPES. All assays were conducted in flat-bottomed 96-well microtitre plates. Cultures were incubated for 24 h at 37 °C in a humidified 5% CO₂ atmosphere with the addition, in 20 μl of DMEM, of 1·0 μCi of [Me³H]thymidine ([³H]TdR; Amersham) during the final 8 h. Cells were harvested with a MASH harvester and [³H]TdR incorporation was quantified using a Beckman LS 8000 liquid scintillation counter. The incorporation of [³H]TdR was expressed as mean c.p.m. per culture.

**Electron microscopy.** One × 10⁷ to 3 × 10⁷ BTLs were plated in 35 mm wells and cultured in 4 ml of RPMI 1640 supplemented with 10% FBS and 5 units/ml of rBolL-2. Virus-infected cultures received...
BHV-1 at an m.o.i. of 1 or 2. At various times after infection, control and virus-infected BTLs were collected, washed twice in medium and the cell pellet was fixed in 2.5% glutaraldehyde in 0.1 M-PBS buffer pH 7.1. Further processing for electron microscopy was as described by Bielefeldt Ohmann & Bloch (1982) except that embedding was done in Epon. Ultrathin sections, contrasted with uranyl acetate and lead citrate, were examined in a Philips 410 electron microscope at 60 kV.

**Infection and labelling of cells.** The protocol used to label viral proteins expressed in either MDBK cells or BTLs was adapted from Misra et al. (1981). Briefly, monolayers of approximately 10^7 to 2 x 10^7 MDBK cells or a suspension of 2 x 10^7 BTLs were infected with BHV-1 at an m.o.i. of 2 p.f.u. After the virus adsorbed for 1 h at 37 °C, the cells were washed three times with MEM and then cultured in methionine-free MEM containing 2% FBS. The BTC medium was supplemented with 5 units/ml of rBoIL-2. Replicate cultures were incubated for either 1 or 6 h and then labelled for a further 6 h with 25 μCi of [35S]methionine (Amersham) per ml. The cells were harvested, washed twice with 5 ml of MEM and cell extracts were prepared by the method of Purifoy & Powell (1976).

**Analysis of proteins by SDS-PAGE.** Cellular proteins were separated by SDS-PAGE on 10% slab gels (Laemmli, 1970). This was followed by fluorography using 3M film and incubation at −70 °C for various time periods.

**Statistical analysis.** All virus-induced changes in LPR and cell number were compared with uninfected culture values using a two-way, one-tailed Student's t-test assuming equal variance of the populations.

**Results**

**Effect of BHV-1 on BTL proliferation responses**

The effect of increasing m.o.i. of BHV-1 on BTL viability and proliferation responses was determined. For these assays the BTLs were cultured with BHV-1 for 24 h and then washed four times with 5 ml DMEM before plating cells in 24 h LPR assays. The data from three separate experiments are presented in Fig. 1. A significant decrease in rBoIL-2-induced LPR was observed consistently with an m.o.i. of 5 or greater. Therefore, all subsequent experiments were conducted using an m.o.i. of 5.

To determine whether BHV-1-induced lymphocytolysis was responsible for reduced [3H]TdR incorporation the number of viable cells was quantified following incubation with BHV-1. For these experiments, 20 x 10⁶ BTLs were plated in 100 mm Petri dishes with 16 ml of DMEM containing 5% FBS and 10% CA-CM. Duplicate cultures of BTLs were infected with BHV-1 (m.o.i. of 5) at time zero and cells were collected and counted at 24 h intervals for 3 days. The number of viable BTLs (trypan blue dye exclusion) recovered at each time point was determined for two cultures.

Fig. 2 demonstrates that there was a significant reduction in the number of viable BTLs recovered within 24 h post-infection (p.i.) and few viable BTLs remained...

**Fig. 1.** Suppression of the rBoIL-2-induced LPRs of BTLs cultured with increasing m.o.i. of BHV-1. Significant suppression of the LPRs was observed when the m.o.i. was 5 or greater (P < 0.05). Data are presented as the mean and s.d. of values from three experiments.

**Fig. 2.** The recovery of viable BTLs following cultivation in the absence of virus [control (●)], with infectious BHV-1 [m.o.i. of 5 (○)], and u.v.-irradiated virus [equivalent to an m.o.i. of 5 (△)]. Incubation with infectious virus resulted in significantly reduced cell numbers at 24, 48 and 72 h p.i. (P < 0.01). Data are presented as the mean and s.d. of values from four experiments.
at 72 h p.i. These data indicated that lymphocytolysis was a major factor contributing to decreased [3H]Tdr incorporation.

Viral replication in BTLs

As BHV-1 could induce lysis of BTLs it was of interest to determine whether the infection was productive or abortive. For these experiments, $2 \times 10^6$ BTLs were incubated with BHV-1 (m.o.i. of 5) and an equivalent amount of virus was plated in medium alone. Infectious virus present in these cultures was quantified by plaque assay. The total amount of infectious virus was determined by disrupting cells with a 30 s sonication. The zero time point sample was collected 1 h after plating virus with BTLs. All samples were stored at $-70 ^\circ C$ until assayed. The 'medium control' provided baseline values for the amount of infectious virus that persisted in culture supernatants, and survived sonication, freezing and thawing.

Fig. 3 indicates that there was no significant difference in the level of infectious BHV-1 recovered from cultures with or without BTLs. Therefore, there was no detectable viral replication associated with BTL lysis.

Characterization of the interaction between BHV-1 and BTLs

The specificity of the BHV-1-induced destruction of BTLs was determined by pre-incubating infectious BHV-1 with heat-inactivated rabbit antisera specific for BHV-1. The equivalent of $1 \times 10^7$ p.f.u. of BHV-1 was incubated for 1 h, at $37 ^\circ C$, with 4 neutralizing units of antiserum before the virus–antibody complex was added to $2 \times 10^5$ BTLs. Following a 48 h incubation, the rBoIFN-α1-induced LPRs of the BTLs were assayed. Pre-incubation of the virus with specific antisera completely blocked the virus-induced decrease in the LPR (Fig. 4). Furthermore, viable virus was required since incubation of BTLs with u.v.-irradiated virus did not result in decreased LPR.

The requirement for viable virus suggested that transcription and translation of viral genes may also be integral to BHV-1-induced lymphocytolysis. Therefore, it was postulated that blocking viral gene expression should prevent BTL lysis. Previous investigations indicated that IFN-α could inhibit the replication of BHV-1 in macrophages (Bielefeldt Ohmann et al., 1984) and tracheal epithelium (Fulton & Root, 1978) and this suggested that IFN-α may prevent virus-induced lysis of BTLs. A concentration of $1 \times 10^4$ units/ml of rBoIFN-α1 was used since this was not anti-mitotic (Fig. 4) but had been previously shown to reduce BHV-1 replication (Babiuk et al., 1985). However, when this concentration was included in the culture medium it did not alter the BHV-1-induced decrease in LPR (Fig. 4).
Fig. 5. Autoradiograph of [3S]methionine-labelled proteins, separated by SDS-PAGE. Lane 1, mock-infected BTLs (12 h p.i.); lane 2, BTLs 6 h p.i.; lane 3, BTLs 12 h p.i.; lane 4, mock-infected MDBK cells (12 h); lane 5, MDBK cells 6 h p.i.; lane 6, MDBK cells 12 h p.i. Virus-specific proteins are indicated by arrows in the right margin. None can be distinguished in the BTLs (lanes 1 to 3).

PAGE analysis of BHV-1 proteins

The possibility remained that despite an absence of viral replication there was limited production of viral proteins. However, by autoradiography (Fig. 5) of PAGE-separated cytoplasmic proteins it was not possible to detect virus-specific protein synthesis within BTLs. In contrast, the viral infection proceeded as expected (Misra et al., 1981) in simultaneously infected and processed MDBK cells (Fig. 5). Not only were viral proteins present in MDBK cells but there was also a decreased production of host cell proteins. The latter decrease was in contrast to the constant level of cell proteins present within BHV-1-infected BTLs.

Ultrastructure of BHV-1-infected BTLs

Although virus replication was not detected within BTLs, ultrastructural changes in the cells became evident 4 h p.i. and progressed rapidly through a sequence of events comprising marked lobulation of the nucleus (Fig. 6a) and accumulation of glycogen (Fig. 6b), chromosomal dispersion and fragmentation, pyknosis, karyorrhexis and nucleolysis (Fig. 6c). There was also extrusion of nuclei and/or the formation of cytoplasmic blebs resulting in akaryotic cytoplasmic bodies. The nuclear changes were followed later by changes in the structure of cytoplasmic organelles such as swelling and vacuolation of mitochondria. This mode of cell death differed significantly from that of cell death due to normal senescence. In the latter case cytoplasmic changes preceded nuclear changes (Fig. 7b). Despite an extensive search there was no evidence of early nuclear particle formation, capsid assembly or envelopment (i.e. no precursor particles) in the BHV-1-infected cells.

The effects of BHV-1 on BTLs was characterized as ‘toxic’, the degree of which did not, at the cellular level, have any direct correlation to the m.o.i. That is, the effects were seen at all multiplicities tested, but the number of cells affected at any particular time did depend on m.o.i.

Discussion

The present investigation demonstrates that BHV-1 infection of T lymphocytes caused cytolysis and consequently decreased the LPRs of activated BTLs. These observations were made with a highly pure (>97% BoT2+) bulk culture of BTLs. The BTLs were rapidly proliferating cells which cannot be compared to resting, circulating T lymphocytes. However, these lymphocytes may be more representative of the T lymphocytes localized at the site of infection and involved in virus clearance.

The specificity of the interaction between BHV-1 and BTLs was apparent in, firstly, the dose responsiveness of decreased LPR with increasing m.o.i. of BHV-1 (Fig. 1), secondly, in the time dependence of virus-induced lymphocytolysis (Fig. 2), thirdly, in the inhibition of the virus-induced decrease in LPR with specific antisera (Fig. 4) and finally, the dependence of the virus-induced
Fig. 6. Electron micrographs of BTLs at various times after infection with BHV-1 at an m.o.i. of 1 or 2. Cytoplasmic effects of infection include (a) 'multinucleation', (b) glycogen accumulations [the inset shows input virus 5 min p.i. (× 14000 enlargement)], (c) karyorrhexis, pyknosis, karyolysis and finally plasmolysis and membrane rupture. Photographs (a) and (b) represent 6 h p.i.; (c) was 8 h p.i. Bars represent 1 μm.
Fig. 7. Transmission electron micrographs of uninfected BTLs 8 h after subcultivation and restimulation with rBoIL-2. (a) An intact, healthy cell displaying a nucleus with dispersed chromatin and a ribosome-rich cytoplasm with many organelles. (b) At any time during normal cell cycling a small number of cells will die, a process characterized by chromatin condensation and plasmolysis with the latter process progressing faster than the former. Bars represent 1.5 μm.

The decrease in LPR in the presence of viable BHV-1 (Fig. 4).

The present observations are consistent with a previous investigation (Plaeger-Marshall & Smith, 1978b) which determined that HSV-1-induced lymphocytolysis required viable virus. However, the absence of BHV-1 replication is in contrast to the findings of Hammer et al. (1982) that there was limited HSV-1 replication within all activated human T lymphocyte subsets.

BHV-1-induced lymphocytolysis differed from that reported for HSV-1 in several other aspects. The requirement for viable BHV-1 suggests that a mechanism similar to the secondary phase of HSV-1 shutoff of host cells may be involved (Fenwick & Clark, 1982; Kwong et al., 1988). This would imply that limited expression of viral genes, at least, was essential for BTL lysis. However, data from PAGE (Fig. 5) indicated that viral proteins were not translated and host cell macromolecular synthesis was not inhibited. Furthermore, the nuclear disintegration in BHV-1-infected BTLs (Fig. 6) suggested that events within the nucleus rather than in the cytoplasm were responsible for cell death. This cell death has the character of apoptosis, an active process of cell destruction which requires energy and macromolecular synthesis (Wyllie, 1981; Wyllie et al., 1980). Consistent with this process of cell death was the continued production of cellular proteins in BTLs following BHV-1 infection (Fig. 5). Furthermore, the investigations of Mittnacht et al. (1988) indicated that IFN-α inhibited the transcription of HSV-1 immediate early genes. If rBoIFN-α1 has a similar action this would suggest that BHV-1-induced lymphocytolysis involves a mechanism independent of viral gene transcription or protein synthesis. However, the absence of lymphocytolysis when BTLs were incubated with u.v.-irradiated virus suggests that a virus–receptor interaction did not provide a signal which could disrupt lymphocyte function.

The possibility remains that restricted viral gene transcription and/or translation occurred in BTLs and viral proteins were not detected with the methodology used. However, the development of BHV-1-specific
recombinant DNA probes will be necessary to determine with greater sensitivity whether BTL infection is associated with limited viral gene transcription.

The possible in vivo significance of the present observations is limited by the criterion that BHV-1 must contact activated T lymphocytes. Previous investigations determined that following BHV-1 infection there was rarely infectious virus present in blood (Yates, 1982) and resting, peripheral blood T lymphocytes bound low concentrations of BHV-1 virions (Splitter & Eskra, 1986). These observations argue against a direct interaction between BHV-1 and circulating T lymphocytes as a possible explanation for the lymphopenia frequently associated with BHV-1 infections (Yates, 1982). However, there are reports of lymphoid depletion with virus isolation and viral antigen associated with secondary lymphoid organs following multisystemic BHV-1 infections of neonatal calves (Mechor et al., 1987) or enteric BHV-1 challenge (Peter et al., 1966). Therefore, viral infection and lysis of activated T lymphocytes within either secondary lymphoid organs or a population of tissue infiltrating lymphocytes may be a significant component of the pathogenesis of BHV-1 disease. The destruction of T lymphocytes could occur even in the presence of virus-induced IFN-α.

Tissue-infiltrating T lymphocytes play an important role in the clearance of local herpesvirus infections (Nash et al., 1987; Schmid, 1988). Therefore if localized T lymphocytes were destroyed by BHV-1 virus clearance could be delayed. Also, the destruction of activated T lymphocytes could have much broader effects on the host immune response if the local T lymphocyte population includes cells with a range of antigen specificities. This may be another mechanism by which BHV-1 infection results in a non-specific suppression of pulmonary defences.

In conclusion, activated BTLs are susceptible to BHV-1 infection and lysis in the absence of viral replication or the expression of detectable levels of viral proteins. This lymphotolysis requires viable virus and is resistant to IFN-α.1. An evaluation of the significance of these findings, in terms of local immunity, will require a direct investigation of the function and fate of T lymphocytes localized at the site of viral infection.

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