Tropism of Varicella-Zoster Virus for Human Tonsillar CD4⁺ T Lymphocytes That Express Activation, Memory, and Skin Homing Markers

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Received 3 June 2002/Accepted 19 August 2002

Varicella-zoster virus (VZV) is an alphaherpesvirus with the characteristic neurotropism of this group, but VZV also infects T cells productively and downregulates major histocompatibility complex (MHC) class I expression on infected T cells, as shown in the SCID-hu mouse model. T-cell tropism is likely to be critical for the cell-associated viremia associated with primary VZV infection. In these experiments, we found that VZV infects human tonsillar CD4⁺ T cells in culture, with 15 to 25% being positive for VZV proteins as detected by polyclonal anti-VZV immunoglobulin G (IgG) staining and monitored by flow cytometry analysis. RNA transcripts for VZV gE, a late gene product, were detected in T-cell populations that expressed VZV surface proteins, but not in the VZV-negative cell fraction. Exposure to phorbol myristate acetate resulted in an increase in VZV-positive T cells, indicating that viral DNA was present within these cells and that VZV gene expression could be induced by T-cell activation. By immune scanning electron microscopy, VZV virions were detected in abundance on the surfaces of infected tonsillar T cells. The predominant CD4⁺ T-lymphocyte subpopulations that became infected were activated CD69⁺ T cells with the CD45RA⁺ memory phenotype. Subsets of CD4⁺ T cells that expressed skin homing markers, cutaneous leukocyte antigen, and chemokine receptor 4 were also infected with VZV. By chemotaxis assay, VZV-infected T cells migrated to SDF-1, demonstrating that skin migratory function was intact despite VZV infection. The susceptibility of tonsil T cells to VZV suggests that these cells may be important targets during the initial VZV infection of upper respiratory tract sites. Viral transfer to migrating T cells in the tonsils may facilitate cell-associated viremia, and preferential infection of CD4⁺ T cells that express skin homing markers may enhance VZV transport to cutaneous sites of replication.

Varicella-zoster virus (VZV) is a human alphaherpesvirus that has a linear double-stranded DNA genome consisting of approximately 125,000 bp and encoding at least 69 unique proteins (34). VZV is a highly contagious pathogen. Primary infection leads to varicella (chickenpox) in susceptible individuals and establishment of latency in sensory ganglia; VZV may reactivate as herpes zoster (shingles) (3). Healthy individuals acquire VZV through contact with skin lesions of an infected person, followed by inoculation of respiratory mucosa, or by direct inoculation of virus in respiratory secretions. By analogy with mousepox, VZV has been presumed to be transferred to regional lymph nodes, where it is thought to initiate a primary viremic phase within a few days after inoculation (12). The mechanism of VZV transport from respiratory mucosa to regional lymph nodes is not known. A secondary viremia has been demonstrated to occur at the end of a 10- to 21-day incubation period, accompanied by the appearance of skin lesions. VZV has been recovered from cultured mononuclear cells obtained from 5 days before until 2 days after the appearance of a rash in children with varicella (4, 27), and VZV DNA can be detected in peripheral blood lymphocytes by *in situ*

hybridization or PCR (17, 18, 35). Our experiments with SCID-hu mice with human fetal thymus or liver implants demonstrated that VZV DNA is present extensively within thymic lymphocytes, and viral protein synthesis is evident in all T-cell subpopulations, including CD4⁺ or CD8⁺ single-positive T cells as well as CD4⁺ CD8⁺ double-positive cells (24). Furthermore, the release of infectious virus from T cells obtained after 7 days of VZV replication in thymus/liver implants suggests that circulating infected T cells could disseminate newly synthesized virions to the skin, which is the second site important for VZV pathogenesis during primary infection.

VZV is highly cell associated when grown in cell culture. No primary cells or cell lines support the replication of VZV to release significant amounts of cell-free virus. Accordingly, the stages of VZV replication cannot be analyzed by standard virologic methods, because a synchronous VZV infection cannot be induced. However, it is likely to follow the typical herpesvirus pattern of immediate-early, early, and late gene expression. During infection, the envelope glycoproteins of VZV are presumed to mediate viral attachment to host cells (28, 45), followed by the release and transport of viral tegument proteins to the nucleus, where they may serve to activate transcription of viral genes (16, 23, 29, 30).

T cells are infectible with VZV in culture. However, the low level of infection in cord blood-derived primary T cells (18, 36)

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and abortive infection in T-cell lines (44) suggest that the growth of VZV is inefficient in resting, quiescent T cells or is restricted to some T-cell populations. In this paper, we report that T cells isolated from human tonsils were readily infected with VZV *in vitro* and produced infectious virions. We demonstrated that VZV had increased tropism for T cells of the activated, memory phenotype with skin homing markers.

MATERIALS AND METHODS

Virus and cell culture. Cosmid-derived vaccine Oka virus (V-Oka) was made as described previously (14, 22). Recombinant V-Oka was recovered from melanoma cells and propagated in human embryonic lung (HEL) fibroblasts in tissue culture medium containing minimal essential medium supplemented with 50 IU of penicillin, 50 mg of streptomycin, and 0.5 mg of amphotericin B (Fungizone) (Flow Laboratories, Inc., McLean, Va.) plus 10% fetal bovine serum (FBS). After three passages in HEL cells, V-Oka stocks were stored in freezing medium with 10% dimethyl sulfoxide (DMSO) at -70°C ; V-Oka was grown in HEL cells for transfer to T cells and used at a maximal passage of 12.

Infection of human T cells. Human tonsil tissue was obtained according to a protocol approved by the Stanford University Committee for Research Involving Human Subjects. The tonsils were dissociated to single cells, resuspended in prewarmed RPMI-10% FBS, and incubated at 37°C for at least 30 min to remove adherent cells. Nonadherent cells were loaded on a sterile nylon wool column that had been washed and soaked in Hanks' balanced salt solution (HBSS)-5% FBS at 37°C and then were incubated for 30 to 40 min at 37°C , and cells were eluted in an appropriate volume of HBSS-5% FBS. The eluted cells were stored in freezing medium with 10% DMSO at -70°C . To further purify T cells, the cells recovered from nylon wool columns were loaded onto an affinity T-cell column (Pierce, Inc., Rockford, Ill.) and eluted according to the manufacturer's protocol; the purity of T cells was $>95\%$.

Infection of T cells with VZV was achieved as previously described (44). Briefly, 5×10^6 to 10×10^6 column-purified T cells were resuspended in 7 ml of RPMI-10% FBS supplemented with 4 IU of interleukin-2 (IL-2), 50 mM β -mercaptoethanol, and 10 μg of gentamicin per ml (GIBCO, Inc., Rockville, Md.). T cells were added to a monolayer of V-Oka-infected HEL cells in a T-75-cm² flask when the HEL monolayer exhibited a cytopathic effect (CPE) of 1 to 2 and incubated at 37°C for 2 to 3 days with daily changes of medium. T cells were then removed from the flask and washed before further analysis. In some experiments, peripheral blood T cells were used instead of tonsil T cells.

To stimulate VZV replication in infected T cells, T cells cocultured with a VZV-infected HEL monolayer were removed and incubated with the phorbol ester phorbol myristate acetate (PMA; Promega, Inc., Madison, Wis.) at 100 nM at 37°C for 48 h. The cells were also incubated with a mitogen-activated protein kinase (MAPK) inhibitor, U0126 (Promega, Inc.), at 10 μM in 0.1% DMSO at 37°C , 30 min prior to PMA treatment. Cells from the same preparation were incubated with 0.1% DMSO as negative controls for U0126 treatment.

Antibodies and flow cytometry analysis and separation. Phycoerythrin (PE)-, fluorescein (FL)-, CyChrome (CyC)-, PerCp-, and allophycocyanin (APC)-labeled antibodies for human cell surface proteins were used in four-color analysis. These monoclonal antibodies (MAbs) included anti-CD3 (clone UCHT1); anti-CD4 (clone RPA-T4); anti-ICAM-1 (anti-CD54, clone HA58); anti-transferrin receptor (anti-CD71, clone M-A712), and anti-integrin 7 (clone FIB504) (BD Pharmingen, Inc., San Jose, Calif.). Additional PE- or FL-labeled MAbs included anti-CD25 (clone CD25-3G10), anti-CD38 (clone HIT-2), anti-CD45RA (clone MEM56), anti-CD57 (clone TB01), anti-CD62L (clone DREG-56), and anti-CD69 (clone CH14) (Caltag, Inc., Burlingame, Calif.). Antihuman chemokine receptor 4 (CCR4) MAbs (clone 1G1) was a gift from David Andrews (LeukoSite, Inc., Cambridge, Mass.). FL-conjugated antihuman CLA (clone HECA-452) was kindly provided by Eugene Butcher, Stanford University. Isotype control MAbs for mouse immunoglobulin G1 (IgG1) (BD Pharmingen, Inc.), mouse IgG1, and mouse IgG2a (Caltag, Inc.) were included in fluorescence-activated cell sorter (FACS) analysis.

T cells (10^6) were resuspended in 100 μl of staining buffer (phosphate-buffered saline [PBS]-2% FBS-0.1% sodium azide) containing 10% normal mouse IgG (Sigma, St. Louis, Mo.). The cells were then incubated with directly conjugated MAbs on ice for 30 min. For CCR4 staining, the cells were resuspended in staining buffer and incubated with 1 μg of anti-CCR4 MAbs on ice for 30 min. The cells were washed and incubated with PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa.) for another 30 min on ice followed by addition of other fluorochrome-conjugated MAbs to human

T-cell surface markers. VZV-infected T cells were detected with IgG-purified polyclonal anti-VZV human immune serum, which binds primarily to viral glycoproteins and fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Caltag, Inc.) followed by staining with a panel of MAbs against human surface proteins and CD3. Normal human serum with no VZV IgG was used as a control for background staining. The stained cells were analyzed by FACS-Calibur with CellQuest software (Becton Dickinson, Inc., San Jose, Calif.). Subpopulations of virus-infected and uninfected T cells were separated by FACS-Vantage (Becton Dickinson, Inc.).

RNA extraction and RT-PCR. T cells were infected with VZV as described above and stained with PE-conjugated anti-human CD3 for FACS. After sorting, 5×10^6 to 10×10^6 CD3⁺ T cells were homogenized by incubation with 1 ml of Trizol reagent (Invitrogen, Inc., Carlsbad, Calif.) for 5 min at room temperature. Chloroform (200 μl) was added to the homogenized samples, which were then centrifuged at 12,000 rpm in a Sorvall Biofuge fresco (Kendro Laboratory Products, Newtown, Conn.) for 15 min at 4°C . RNA from the aqueous phase was precipitated with isopropanol alcohol and washed twice in 70% ethanol by centrifugation at 10,000 rpm for 5 min at 4°C . The RNA pellet was briefly air dried, resuspended in diethyl pyrocarbonate-water, and stored at -20°C .

VZV gE transcripts were analyzed by reverse transcription-PCR (RT-PCR) with the SUPERScript One-Step RT-PCR with PLATINUM *Taq* system (Invitrogen, Inc.) according to the manufacturer's instruction. Prior to cycling amplification, DNA contaminating the RNA sample was removed by incubating 10 ng of template RNA with 5×10^4 cell equivalents with 0.1 U of DNase I (amplification grade; Invitrogen, Inc.) at room temperature for 15 min. The DNase I was inactivated by adding 1 μl of 25 mM EDTA to the mixture and heating at 65°C for 10 min. The mixture in a 50- μl reaction mixture containing 200 nM sense and antisense primers plus 2 U of RT/PLATINUM *Taq* mix and 2 $\times$ reaction mixture was amplified as follows: 1 cycle of 50°C for 30 min and 94°C for 2 min for cDNA synthesis and predenaturation; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and, finally, 1 cycle of 94°C for 30 s, 55°C for 30 s, and 72°C for 5 min. For the RNA control, the RT/*Taq* mix was replaced with 2 U of *Taq* mixture and amplified as described above. The oligonucleotide primer sequences of gE in the VZV Spe23 cosmid were as follows: sense, 5'-GCGCA CCGATTACAG; and antisense, 5'-CCATGCGGCATATCGTA.

Infectious focus assay. T cells recovered from V-Oka-infected HEL cells were stained with anti-VZV immune serum and anti-CD3 MAbs and separated into infected and uninfected CD3⁺ T-cell populations by cell sorting. The cells were counted, inoculated on a subconfluent monolayer of melanoma cells in 12-well plates, and incubated at 37°C for 5 to 7 days until CPE was observed. The monolayers were fixed in 4% paraformaldehyde, washed in PBS, and incubated with anti-VZV immune serum at 1:500 for 1 h at room temperature. Infectious foci were detected by adding biotinylated antihuman IgG (Vector, Inc.) for 30 min at room temperature and washing and incubation with streptavidin-alkaline phosphatase reagent (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) for 30 min at room temperature. Plaques were visualized by adding Fast Red substrate (Sigma, Inc.) and counted.

Chemotaxis assay. Chemotaxis assays were performed with 24-well-plate tissue culture inserts (Costar, Inc., Cambridge, Mass.) with 5- μm -pore-diameter polycarbonate filters. Recombinant human chemokine SDF-1 α was purchased from PeproTech, Inc. (Rocky Hill, N.J.). T cells were cocultured with V-Oka-infected HEL cells and removed from the infected monolayer 2 days after infection. The T cells were washed in PBS and resuspended in chemotaxis medium, consisting of RPMI-0.5% bovine serum albumin (BSA). T cells (5×10^5 cells in 100 μl) were placed inside the insert, and 600 μl of a given chemokine dilution was added to the bottom well. The plate was incubated at 37°C for 2 h, and the inserts were removed. The cells that had migrated were harvested and analyzed for their expression of T-cell surface markers by FACS. The numbers of a particular T-cell subset that were present in the original and the migrating populations were normalized. The percentage of T cells that migrated to the SDF-1 α chemokine was calculated, and results were expressed as a percentage of maximal migration (8).

Electron microscopy. Infected and uninfected T lymphocytes were fixed in 0.1% glutaraldehyde in sodium phosphate buffer (PBS) at pH 7.4 for 10 min. Samples were then pelleted down at 1,000 rpm for 15 min; lymphocytes were resuspended in PBS and gently rinsed. Resuspended lymphocytes were then blocked with 5% BSA and 1% normal goat serum for 30 min and incubated with MAb 3B3 at 37°C for 1 h. Rinsed samples were then incubated with a secondary antibody (goat anti-mouse IgG coupled to 10-nm-diameter colloidal gold particles) diluted 1:50 in PBS at 37°C for 1 h, followed by extensive washes with PBS. Immunogold labeling samples were fixed with 1% glutaraldehyde-PBS for 1 h at 4°C and rinsed with chilled PBS, followed by postfixation with 1% osmium tetroxide in PBS at 4°C for 1 h. Samples were mounted in a poly-L-lysine-

FIG. 1. FACS analysis of VZV-infected CD3⁺ T cells. Column-purified T cells isolated from tonsils were cocultured with VZV-infected HEL monolayers. The T cells were analyzed at days 2 to 3 postinfection and stained with anti-CD3 MAb and VZV IgG-negative human serum (A) as background staining or with anti-VZV IgG polyclonal immune serum (B) to detect VZV glycoproteins, which provide the indication of infection. The FACS plots show infection of tonsil T cells with VZV, and the frequency of CD3⁺ T cells that are VZV positive is as indicated.

covered glass coverslip, dehydrated in a graded ethanol series, and dried by a critical point drying method. Samples were then mounted onto aluminum plates and were observed in a high-resolution scanning electron microscope (LEO5300).

RESULTS

Expression of VZV proteins on human tonsil T cells. T cells isolated directly from tonsils were infected with VZV as judged by surface expression of VZV proteins. In more than 10 experiments, when column-purified T cells from human tonsils were incubated with VZV-infected HEL monolayers, viral proteins were detected on surfaces of 15 to 25% of CD3⁺ T cells incubated with polyclonal VZV IgG serum and examined by flow cytometry. A representative experiment is shown in Fig. 1. Both CD4⁺ and CD8⁺ T cells from tonsils were infected by VZV. The percentages of VZV infection from four experiments were 20.5% (range, 11 to 28%) for CD4⁺ T cells and 13% (range, 8 to 22%) for CD8⁺ T cells. More than two-thirds of CD3⁺ T cells recovered from tonsils were CD4⁺ T cells. For subsequent experiments, we focused on VZV-infected CD4⁺ T cells. In contrast to CD4⁺ T cells from tonsils, VZV infection was detected in 4 to 10% of CD4⁺ T cells from peripheral blood from four experiments (data not shown).

VZV gene transcription and production of infectious VZV by CD4⁺ T cells. Flow cytometry analysis of VZV infection detected late viral proteins on T-cell membranes, indicating that viral genes of all three classes were transcribed. However, we have observed that VZV infection of the CD4⁺ T-cell hybridoma cell line, designated II-23, progressed to late gene expression, but no infectious virus was produced (44). To demonstrate that VZV replicated in T cells, RT-PCR was used to detect *de novo* transcription of the most abundant late gene, gE, as shown in Fig. 2. RNA isolated from VZV-infected HEL fibroblasts was included as positive control for gE (Fig. 2, lanes 1 and 2). CD3⁺ T cells were separated by FACS after coculture with VZV-infected HEL to remove infected fibroblasts that served as an inoculum. Thirty percent of these cells were positive for gE expression by staining with anti-VZV IgG immune

FIG. 2. Detection of VZV gE transcripts by RT-PCR. Ten nanograms of template RNA isolated from VZV-infected fibroblasts (lanes 1 and 2), VZV-infected and FACS-sorted CD3⁺ T cells (lanes 3 to 6), or uninfected T cells (lane 7) was amplified by RT-PCR in the presence (lanes 1, 3, 5, 7, and 8) or absence (lanes 2, 4, and 6) of reverse transcriptase. The RNA was treated (lanes 1, 2, 5, 6, and 7) or untreated (lanes 3 and 4) with DNase I prior to cycling amplification. A 1,070-bp fragment for VZV gE was detected as indicated. Lane 8, no-template control (NC).

serum. A 1,070-bp fragment for VZV gE was amplified by RT-PCR in CD3⁺ T cells (50,000 cells per sample) that had been cocultured with VZV-infected HEL monolayer (Fig. 2, lanes 3 and 5), but not in uninfected CD3⁺ T cells (Fig. 2, lane 7). Although a small amount of viral DNA was present in the CD3⁺ RNA sample, it was eliminated by DNase I treatment (Fig. 2, lanes 4 and 6). The results indicated that RT-PCR amplified mRNA representing gE gene transcription. The presence of gE transcripts in the infected CD3⁺ T cells suggested that VZV progressed through all stages of viral replication in T cells after entry and indicated that expression of VZV proteins on the T-cell surface was due to intracellular viral synthesis rather than residual virions from the inoculum.

To further demonstrate that the T cells were productively infected with VZV as opposed to exhibiting surface attachment of virions, T cells were stimulated with PMA after coculture with a VZV-infected HEL monolayer. The expression of VZV glycoproteins by T cells was analyzed by FACS. Results from one of three such experiments are shown in Fig. 3. VZV replication in T cells was enhanced after PMA activation, since the percentage of CD3⁺ T cells that expressed VZV proteins increased from 17.8% in mock-treated T cells (Fig. 3A) to 43.5% in PMA-treated T cells (Fig. 3B). Furthermore, this enhancement was inhibited by U0126 (Fig. 3C), a MAPK inhibitor that blocks downstream proteins mediated by PMA activation (9). The data suggested that VZV glycoproteins expressed on T cells detected by FACS were due to transcription of VZV genomic DNA present within T cells prior to PMA activation. The results, together with RT-PCR analysis, demonstrated that VZV actively infected T cells.

FIG. 3. PMA stimulation of VZV transcription in virally infected T cells. VZV-infected T cells at 2 days postinfection were preincubated either with 0.1% DMSO alone (A and B) or with U0126 at 10 μ M in 0.1% DMSO (C) at 37°C for 30 min followed by PMA stimulation at 100 nM at 37°C for 24 h. The T cells were washed and stained with antihuman CD3 MAb and anti-VZV IgG immune serum for FACS analysis. The FACS plots show that VZV replication indicated by expression of VZV glycoproteins was enhanced by PMA treatment (B), but was inhibited by U0126 (C) compared to the unstimulated control (A).

To determine whether primary CD4⁺ T cells from tonsils were productively infected, VZV-infected T cells were tested with the infectious focus assay. VZV-infected T cells were recovered from infected HEL cell monolayers, sorted, and inoculated onto melanoma cells. One plaque per 100, 800, and 5,000 T cells was observed in three experiments in which melanoma cells were fixed and stained 5 to 7 days after inoculation with sorted T cells that expressed VZV protein. No plaques were observed in monolayers inoculated in parallel with the VZV-negative T cells from each cell preparation. The results demonstrated that CD4⁺ T cells were permissive for VZV replication and that infectious VZV was transferred from T cells to other permissive cells.

Detection of VZV virions on CD3⁺ T-cell membranes by immunogold imaging. Column-purified T cells, >95% of which were CD3⁺, were cocultured with a VZV-infected HEL monolayer and isolated by Ficoll centrifugation. T cells from the same preparation that did not express VZV proteins were used as a control. By scanning electron microscopy, the lymphocytes were grossly spherical, with occasional protruding microvilli. No viral particles were expressed on the surfaces of uninfected T cells (Fig. 4A). In contrast, VZV-infected T cells exhibited ruffles and microvilli on their surfaces (Fig. 4B). When viewed at higher magnification, viral particles from 150 to 200 nm in diameter also were visible on the surface of these cells (Fig. 4C). The virions were spherical, and most were found between the microvilli (Fig. 4C); occasionally, virions were also seen in small clusters (Fig. 4B). Virions exhibited specific immunogold labeling of the VZV glycoprotein gE on their envelopes (Fig. 4C). In previous studies with scanning electron microscopy (13), VZ virions were observed to emerge onto the surface of infected human melanoma cells in a distinctive pattern termed “viral highways.” The particles in the highways were pleomorphic in size and shape. In lymphocytes, the particles emerged randomly over the surface and did not form viral highways; however, the particles remained pleomorphic in size.

Relationship between CD4⁺ T-cell memory/naive and activation phenotypes and VZV infection. Cell surface phenotypes of CD4⁺ T cells from tonsils and peripheral blood were examined as background for the analysis of relative susceptibility of

CD4⁺ T-cell subpopulations to VZV infection (Fig. 5). As expected, most CD4⁺ T cells from peripheral blood were CD69[−] (Fig. 5A). In contrast, many tonsillar CD4⁺ T cells were CD69⁺, including naive (CD45RA⁺) as well as memory (CD45RA[−]) subpopulations. The average frequency of CD4⁺ T cells that expressed CD69 was 20% (range, 14 to 38%) for naive cells and 85% (range, 77 to 92%) for memory cells, respectively, in 10 tonsil specimens (Fig. 5B). Constitutive expression of CD69 on T cells in tonsils may be due to chronic exposure to cytokines and antigens in the tissues (19). Expression of transferrin receptor CD71, which is a surface marker for cellular proliferation, was also elevated on tonsillar CD4⁺ T cells (Fig. 5D) compared to CD4⁺ T cells from peripheral blood (Fig. 5C).

When the relative susceptibility to VZV infection of unfractionated CD4⁺ T cells that were CD45RA⁺ or CD45RA[−] was analyzed, memory T cells were consistently more likely to be VZV infected than naive T cells. A representative experiment is shown in Fig. 6. In order to further analyze this phenomenon, experiments were done in which CD4⁺ CD45RA⁺ and CD4⁺ CD45RA[−] T-cell markers were used to separate the populations into naive and memory subsets before VZV infection. When the frequency of VZV-infected T cells was assessed 2 to 3 days after inoculation, memory T cells were significantly more likely to be VZV infected than naive T cells. Although the percentages of T cells that were infected varied in five independent experiments, an increase of at least threefold was observed in memory T cells (paired *t* test, *P* < 0.05) (Table 1). Although it was not likely that VZV expression would alter CD45 isoforms, control experiments were done to show that T cells remained CD45RA⁺ despite being incubated with infected HEL cells (data not shown).

As shown in Fig. 5, most of the memory CD4⁺ T cells in tonsils were CD69⁺, and about 10 to 15% of these T cells also expressed CD71. To further examine the phenotypes of CD4⁺ T cells that were most likely to be infected with VZV, we separated naive and memory T cells into activated and nonactivated subsets, based on the expression of the CD69 activation marker. At 2 days after infection, the percentages of CD69[−] naive, CD69[−] memory, and CD69⁺ memory cells that were infected by VZV were 6, 14, and 24%, respectively. Although

FIG. 4. Expression of VZV particles on infected T cells by scanning electron microscopy. (A) Two uninfected CD3⁺ T cells were visualized by scanning electron microscopy at a magnification of $\times 10,000$. (B) VZV-infected T cells had a more irregular surface than was seen in uninfected T cells. Both ruffles and microvilli, as well as darkened crypts, were abundant on the surface of a representative VZV-infected T cell. In addition, viral particles were also distinguishable on the surface of the cell at magnification of $\times 15,000$. (C) Pleomorphic virus particles ranging in size from 120 to 200 nm were distributed between microvilli and sometimes formed small clusters on the cell surface. A representative high-magnification ($\times 50,000$) scanning electron micrograph shows two viral particles in the center of panel C, to the right of microvilli; arrows indicate that the envelop of the larger particle was labeled with VZV gE immunogold. Micrographs in reference 14 illustrate comparable VZV infection of human melanoma cells.

FIG. 5. Phenotypic analysis of CD4⁺ T cells from tonsils and peripheral blood. T cells isolated from peripheral blood (PBL [upper panels]) and tonsils (lower panels) were stained with anti-CD4, -CD45RA, -CD69, or -CD71 MAbs for FACS analysis. The cells were gated on CD45RA⁺ CD4⁺ naive (shaded area) and CD45RA⁻ CD4⁺ memory (thick line) cells and analyzed for expression of CD69 (A and B) and CD71 (C and D) in these subpopulations. T cells from the same preparation stained with mouse IgG2a were treated as an isotype control (dashed line).

the frequency of VZV-infected T cells was increased in CD69⁺ naive CD4⁺ T cells compared to cells that were CD69⁻, the percentage was consistently lower than was observed for CD69⁺ memory CD4⁺ T cells.

In order to investigate whether VZV was more likely to be detected in CD4⁺ T cells that were proliferating, memory T cells were separated into subsets based on CD71 expression. In this experiment, the percentages of VZV infection were 31.3% for CD71⁺ CD4⁺ memory T cells and 27.3% for CD4⁺ memory T cells without CD71 expression (data not shown). Taken together, these experiments indicate a hierarchy in the susceptibility of CD4⁺ T cells to VZV infection in which activated memory T cells that express CD69 are the predominant phenotype, and naive, resting T cells are least likely to have VZV proteins detected on cell membranes.

VZV infection of CD4⁺ T cells with skin homing markers and effects on chemotactic function. According to Campbell et al. (8), CCR4 is differentially expressed on CLA⁺ skin-associated (>95%) and CLA⁻ intestinal-associated (~20%) memory CD4⁺ T cells (8). Since the final phase of VZV replication during varicella occurs in skin, we investigated the expression of the skin homing markers CLA and CCR4 on infected CD4⁺ T cells. Results from one representative experiment are shown in Fig. 7. VZV-positive CD4⁺ T cells were consistently more likely to have CLA and CCR4 markers than VZV-negative CD4⁺ T cells. This observation might indicate that CLA⁺ or CCR4⁺ T cells are more likely to be infected with VZV or that VZV infection induces the upregulation of CLA and CCR4 on CD4⁺ T cells. Therefore, CD4⁺ T cells were separated into subsets based on CCR4 expression and incubated with VZV-

FIG. 6. Expression of VZV glycoproteins on CD45RA⁺ or CD45RA⁻ CD4⁺ T cells. VZV infection of T cells was achieved, and the cells were then stained as described in the legend to Fig. 1. The cells were gated on CD4⁺ T cells and analyzed by FACS dot plots. The relationship between CD45RA expression and VZV infection in CD4⁺ T cells shows that more memory than naive CD4⁺ T cells are infected with VZV. The numbers indicated in the upper right corner are the percentages of cells in each of the corresponding quadrants.

infected HEL monolayers. In these experiments, 21% of CCR4⁺ CD4⁺ T cells were infected with VZV compared to 9% of CCR4⁻ CD4⁺ T cells. Infection with VZV did not induce the expression of CCR4 by CCR4⁻ naive CD4⁺ T cells. These results indicate that T cells with skin homing phenotypes are infected preferentially by VZV.

Since expression of skin homing phenotypes might persist while chemotactic function was blocked, we tested the migration of VZV-infected CD4⁺ T cells in response to SDF-1 α by using a standard transwell chemotaxis assay. T cells from the initial population and the cells that had migrated in response to SDF-1 α were analyzed for the expression of VZV proteins and cell surface markers. Results from three independent experiments are shown in Fig. 8. In the absence of the chemokine, the mean percentages of background maximal migration for VZV infected and uninfected T cells were 2.8 and 5.2%, respectively; the percentage of infected cells in the migrated population was similar to that in the starting population. As expected, SDF-1 α induced the migration of naive and memory CD4⁺ T cells (Fig. 8). Furthermore, chemotaxis of VZV-in-

ected CD4⁺ T cells was equivalent to migration of uninfected CD4⁺ T cells exposed to SDF-1 α (Fig. 8). These results indicate that chemotactic function is preserved in VZV-infected T cells and suggest that their skin homing potential is not impaired.

DISCUSSION

Human herpesviruses (HHVs) infect cells of the immune system and modulate immune responses to disseminate, evade host clearance, and establish latency. For example, human cytomegalovirus infects and maintains its latency state in monocytes (41, 42). Epstein-Barr virus infects resting B lymphocytes and drives the infected cells into a differentiation pathway or leads to their elimination via programmed cell death (15, 43). Herpes simplex virus type 1 (HSV-1) infects activated cytotoxic T lymphocytes (CTLs) and destroys them through antigen-dependent fratricide (33), which may enhance virus spread from epithelium, the main cell type producing HSV-1. HHV6 and -7 predominantly infect CD4⁺ T cells and depend on T cells for virion production (6, 10, 20).

VZV tropism for human lymphocytes was suspected based on isolation of infectious virus from the nonadherent fraction of peripheral blood mononuclear cells (PBMCs), as well as the morphology of PBMCs that harbored viral DNA determined by *in situ* hybridization, but a more precise identification of the subpopulation of PBMCs involved in VZV viremia has been hampered for technical reasons (2, 11, 18, 27). Our previous experiments in which thymus/liver implants in SCID-hu mice were infected with VZV showed that the virus replicates very efficiently in both CD4⁺ and CD8⁺ T cells, as well as immature CD4⁺ CD8⁺ T cells *in vivo* (24). These observations have demonstrated that VZV is a lymphotropic virus. Soong et al. (36) also showed that human umbilical cord blood mononuclear cells can be infected with VZV in culture and that the majority of the infected cells are T cells.

HSV, the herpesvirus most closely related to VZV, rarely causes viremia in the immunocompetent host. However, primary VZV infection is characterized by the occurrence of cell-associated viremia, which is followed by the appearance of

TABLE 1. VZV infection of naive and memory CD4⁺ T cells^a

Expt	% of cells infected with VZV in CD4 ⁺ subset:		Memory/naive ratio
	Naive	Memory	
1	5.9	22.6	3.8
2	5.7	13.4	2.4
3	5.0	15.0	3.0
4	14.0	40.7	2.9
5	4.0	14.0	3.5
Mean $\pm$ SE	6.9 $\pm$ 1.8	21.0 $\pm$ 5.2	3.1 $\pm$ 0.25 ^b

^a Column-purified tonsillar T cells were stained with anti-CD4 and anti-CD45RA MAbs and separated into CD4⁺ CD45RA⁺ naive and CD4⁺ CD45RA⁻ memory T cells by cell sorting. The subpopulations were added to a monolayer of V-Oka-infected HEL cells and harvested at 2 days postinfection. The percentages of cells that had been infected with VZV were determined as described in the legend to Fig. 1. In five independent experiments, the mean percentage of memory CD4⁺ T cells that were VZV infected was threefold higher than that of naive CD4⁺ T cells.

^b $P < 0.05$ by paired *t* test.

FIG. 7. Expression of CLA and CCR4 on VZV-infected CD4⁺ T cells. Column-purified T cells from tonsils were infected with VZV as described in the legend to Fig. 1. The T cells were analyzed on day 2 and stained with anti-CD4 MAb, anti-VZV IgG polyclonal immune serum, and anti-CLA or anti-CCR4 antibody for FACS analysis. The cells were gated on uninfected (A) and VZV-infected (B) CD4⁺ T cells and analyzed for CLA and CCR4 expression. The fluorescent profiles of CLA and CCR4 in uninfected (upper panels) and VZV-infected (lower panels) CD4⁺ T cells show more CLA and CCR4 are expressed on VZV-infected than on uninfected CD4⁺ T cells. T cells from the same preparation were stained with mouse IgG1 as an isotype control for CCR4.

widely distributed cutaneous vesicles. Hence, transport by infected lymphocytes could accelerate the spread of VZV through lymphoid and reticuloendothelial tissues, which are considered to be the initial sites of VZV replication during primary infection, to the skin. Transfer of VZV to epidermal cells may be enhanced by release of virus from infected T cells trafficking through skin capillaries. In our experiments, detection of VZV glycoproteins expressed on the surface of tonsillar T cells, as well as the presence of late gene transcripts within these cells, indicated that the virus had entered the T cells and that viral gene transcription had progressed through all three stages required for replication following entry. When these

FIG. 8. Chemotaxis of VZV-infected T cells in response to SDF-1 α . Tonsillar T cells infected with VZV were tested for their chemotactic response to human SDF-1 α (100 nM) with 24-well plate tissue culture inserts with 5- μ m-pore-size filters. All samples were performed in duplicate wells. Cells that were incubated with chemotaxis medium without containing chemokine were treated as negative controls. After 2 h of incubation at 37°C, cells that were in the starting population and that had migrated were harvested and stained with anti-CD4, anti-CD45RA, and anti-VZV IgG immune serum. The cells were gated on uninfected (VZV⁻ CD4⁺) and infected (VZV⁺ CD4⁺) T cells. The percentages of total CD4⁺ T cells (black bars), CD45RA⁺ naive (dotted bars), and CD45RA⁻ memory (hatched bars) CD4⁺ T cells that had migrated to SDF-1 α from starting cell populations (maximal migration) in uninfected and infected cells were thus calculated and subtracted from background migration (range, 2.8 and 5.2%). The results shown are the averages of duplicate samples from three independent experiments.

infected T cells were separated from infected HEL monolayers and cocultured with melanoma cells, viral plaques appeared on days 5 to 7 after inoculation, showing that virions made in T cells had acquired all of the viral elements needed for cell-to-cell spread. Dendritic cells can also be infected productively with VZV in vitro (1), and surface expression of VZV proteins can be detected on Epstein-Barr virus-transformed B cells and macrophages infected in vitro (reviewed in reference 2). Evaluation of the effects of mitogen stimulation on these cell types is of interest based on our observation with VZV-infected T cells.

Memory T cells acquire tissue-selective migratory capabilities after antigenic stimulation (21). They are preferentially recruited to extralymphoid sites of inflammation (7, 21). Memory T cells that express low levels of $\alpha 4\beta 7$ and are CLA⁺ tend to migrate to the skin (7, 8). CLA⁺ memory T cells constitute 10 to 25% of circulating CD3⁺ peripheral blood lymphocytes, 2 to 10% of T cells in tonsils and peripheral lymph nodes, and ~80 to 90% of T cells in most cutaneous sites of chronic inflammation (31). CLA is a carbohydrate ligand for endothelial cell-leukocyte adhesion molecule 1 (ELAM-1), which is selectively expressed on venules of inflamed skin, and functions as skin lymphocyte homing receptor (5). Binding of CLA to ELAM-1 triggers the subsequent attachment and extravasation of circulating lymphocytes from blood into peripheral lymphoid tissues. Most CLA⁺ memory CD4⁺ T cells express high levels of CCR4, which responds to its ligand, thymus-

activation-regulated chemokine (TARC), and macrophage-derived chemokine (32). Immunohistochemistry reveals anti-TARC activity of venules and infiltration of CCR4-expressing lymphocytes in chronically inflamed skin, indicating that TARC and CCR4 are involved in the homing of a major subset of circulating systemic memory T cells (8). In the phenotypic analysis of VZV-infected T cells, we showed that T cells expressing CLA and CCR4 were more likely to be VZV infected. Results from the chemotaxis assay demonstrated that T cells infected with VZV migrated to SDF-1 α , indicating that products of VZV genes did not antagonize T-cell chemotactic responses to endogenous ligands. Hence, a subset of VZV-infected T cells that expressed CLA and CCR4 could likely target skin. Taken together, the data suggested that VZV-infected lymphocytes have the potential to facilitate the spread of virus through lymphoid and reticuloendothelial tissues to skin via the usual mechanism of lymphocyte trafficking.

We demonstrated that T cells isolated directly from tonsils were readily infected with VZV. Our experiments revealed a hierarchy of CD4⁺ T-cell susceptibility to VZV infection. First, CD4⁺ T cells of the activated memory phenotype were more likely to be infected with VZV than resting naive CD4⁺ T cells. The increased susceptibility of memory CD4⁺ T cells is of interest from a clinical perspective, because adults have much more serious varicella than young adults. Second, activated CD69-bearing T cells had a higher susceptibility to VZV infection than the cells that did not express CD69. The differential infectivity in T cells of various phenotypes suggested that changes in expression of surface molecules on activated T cells may have an effect on the susceptibility of T cells to VZV infection. Changes in the components of the cell surface membrane could render activated memory CD4⁺ T cells more susceptible to VZV infection by enhancing expression of an as yet undefined receptor molecule.

T-cell activation is required for productive infection with several viruses. Infection of resting T cells with HHV6 gives no infectious virions, but lytic infection can be induced in infected T cells after phytohemagglutinin activation in tissue culture (10). Although HIV-1 enters resting naive CD4⁺ T cells, subsequent virus replication is blocked at nuclear import of viral DNA and results in a nonproductive infection. In contrast, productive replication is induced after T cells are activated with mitogens or anti-CD3 or anti-CD28 MAbs, possibly via activation of MAPKs (37, 39, 40). VZV IE62 protein, which is the predominant tegument protein and the major VZV-transactivating factor, is required for rapid induction of viral protein synthesis (16, 26, 30). The product of open reading frame 47 (ORF47) is a serine-threonine protein kinase (25, 38) that specifically phosphorylates IE62 to modify its function and nuclear translocation (26). A mutant VZV strain deficient in the ORF47 putative kinase was unable to replicate in the SCID-hu mouse model (24) or in peripheral blood T cells in vitro (36). The block in the infectivity of ORF47-deficient VZV for T cells may be due to impaired IE62 nuclear translocation or the absence of ORF47-mediated phosphorylation of other viral or cellular proteins to induce conditions favorable for viral replication. Our experiments showed that PMA induced virally infected T cells to express VZV glycoproteins, suggesting that T-cell activation might stimulate viral replication, either by enhancing phosphorylation and nuclear trans-

location of IE62 or by transactivating transcription of multiple viral genes. Experiments with PMA suggest that VZV replication in T cells may depend on functions of viral gene products after entry, rather than upon upregulation of an entry molecule on the T-cell surface.

T-cell infection is critical for the viremic phase of VZV pathogenesis. Although lymphopenia is observed in patients during the last few days of the incubation period, the total leukocyte counts rise rapidly, beginning on the 2nd and 3rd days of rash, and viremia is resolved (2). The findings in this analysis of VZV T-cell tropism have led us to hypothesize that VZV may target T cells in tonsils for infection shortly after inoculation of respiratory mucosa. The infected T cells could transfer to regional lymph nodes that contain many T cells of activated phenotypes and then emerge into the circulation, where they need to survive only long enough to reach skin sites of replication. VZV infection of activated, memory T cells, which include the skin-migratory population, may enhance the interaction of infected T cells with capillary endothelium and facilitate transfer of the virus to dermis and epidermis, initiating the cutaneous phase of primary VZV infection.

ACKNOWLEDGMENTS

We thank David Andrews for the anti-CCR4 MAb, James Campbell and Eric Kunkel for assistance with chemotaxis experiments, Leigh Zerbini for technical consultation, and Lusijah Rott for cell sorting assistance.

This research was supported by NIH grants AI20459, AI 22795, and CA49605.

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