Investigation of Varicella-Zoster Virus Infection of Lymphocytes by In Situ Hybridization

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Peripheral blood mononuclear cells harboring viral gene sequences were detected during primary varicella-zoster virus (VZV) infection of the human host and the strain 2 guinea pig by in situ hybridization with a ³H-labeled VZV DNA probe. Activated T lymphocytes were permissive for VZV infection at low frequency in vitro.

Primary infection with varicella-zoster virus (VZV), a human herpesvirus, causes varicella, or chickenpox. Varicella is characterized by widely distributed, discrete cutaneous vesicles that contain infectious VZV, indicating that viremia is critical in the pathogenesis of primary VZV infection. VZV can be isolated from peripheral blood mononuclear cells (PBMC) but, despite prolonged cocultivation of PBMC with permissive cell lines, viremia was detected in only 11 to 22% of subjects with acute varicella who were not immunocompromised (2, 4, 16). Because of the importance of viremia in varicella, animal models of primary VZV pathogenesis that reproduce this event are valuable. A viremic phase was proved in some strain 2 guinea pigs inoculated with VZV (15). In situ hybridization with virus-specific DNA probes provides a method for analyzing herpesviral pathogenesis at the level of the single cell (14). In these experiments, PBMC from 12 otherwise healthy human subjects with acute varicella were separated from heparinized whole blood by Ficoll-Hypaque gradient centrifugation. In situ hybridization was carried out by the method of Brahic and Haase (5), by using a ³H-labeled VZV DNA probe prepared from *Hind*III fragments A, B, C, and D (Fig. 1). A similar ³⁵S-labeled probe demonstrated latent VZV infection of sensory ganglion cells (12).

The fragments were radiolabeled by mixing each DNA fragment (500 ng) with DNase I (2.5 ng) and ³H-labeled deoxynucleoside triphosphates (10 μ M, 20 to 70 Ci/mmol) in 50 μ l of 10 $\times$ nick translation buffer (50 mM Tris [pH 7.4], 10 mM MgCl₂, 1 mM dithiothreitol, 50 μ g of bovine serum albumin per ml) and then incubating at 14°C for 2 h with 5 U of *Escherichia coli* DNA polymerase I. The reaction was stopped by adding 400 mM EDTA (2 μ l) and 20% sodium dodecyl sulfate (2 μ l). Unincorporated deoxynucleoside triphosphates were removed by spin column chromatography, and the labeled fragments were ethanol precipitated and reconstituted in Tris-EDTA buffer. The specific activity of the probe was 4.4×10^7 to 15×10^7 dpm per microgram of DNA. PBMC (1×10^6 to 4×10^6 cells) were centrifuged at $15 \times g$ for 5 min onto glass slides at a concentration of 2×10^5 cells per slide. The slides had been pretreated with Denhardt medium and ethanol-acetic acid, dried, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, and dehydrated in graded alcohols. Equimolar amounts of each of the four *Hind*III fragments of VZV DNA or pBR322 DNA alone were mixed with 20 mM Tris buffer (pH 7.4)-2 mg of

salmon sperm DNA per ml-2 mM EDTA-0.2% Ficoll-0.2% polyvinylpyrrolidone-0.2% bovine serum albumin-1.2 M NaCl-20% dextran sulfate in 1 ml of deionized formamide. After denaturing the DNA by boiling for 2 min and quenching on ice, 4.5 μ l of the mixture was added to each of the slides, which had been preincubated at 37°C in 20 mM Tris buffer with 2 mM CaCl₂ and proteinase K for 15 min followed by three separate rinses in distilled water, 70% ethanol, and 100% ethanol. Hybridization was carried out at room temperature in the dark for 96 h. The slides were washed in 2 $\times$ SSC (SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) at room temperature and at 55°C, dehydrated with 70% ethanol-0.3 M ammonium acetate and with 95% ethanol-0.3 M ammonium acetate, dried, and coated with emulsion. After exposure in the dark at 4°C for 2 weeks, the slides were developed with Kodak D-19 for 3 min, fixed for 3 min, counterstained with Giemsa stain for 20 min and examined by light microscopy. Silver grains observed by light microscopy were evidence of positive hybridization. Binding to viral RNA and DNA was demonstrated by preincubation of the cells with 100 μ g of RNase A and 1 μ g of RNase T₁ for 30 min at 37°C followed by 0.1% diethylpyrocarbonate in 2 $\times$ SSC or 2 U of DNase in 40 mM Tris (pH 8)-10 mM MgCl₂-10 mM NaCl for 1 h at 37°C followed by 2 $\times$ SSC washes and dehydration in graded alcohols (data not shown).

The low grain efficiency of ³H required prolonged exposure to demonstrate hybridization, but the low background allowed optimal detection of the signal in PBMC while no binding of the vector plasmid pBR322 DNA was observed (Fig. 2). Because of the denaturing effect of formamide present in the hybridization mixture, viral DNA as well as RNA was accessible to hybridization with the probe. In contrast to latently infected cells (12), and as anticipated in cells harboring infectious virus, pretreatment of cells with either RNase or DNase reduced but did not abolish the signal; this finding indicated that the VZV probe was hybridizing to both viral RNA or DNA. VZV gene sequences were identified only in PBMC obtained during the 24- to 48-h period immediately after the appearance of the varicella rash (Fig. 2). PBMC containing more than 20 grains were observed in six of eight (75%) subjects. The frequency of positive cells ranged from 0.01 to 0.001% of PBMC. No positive PBMC were observed in four subjects who were evaluated from 48 to 72 h after the onset of the varicella exanthem or in four subjects tested from 10 to 29 days later. While cell-associated viremia could be detected much more

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FIG. 1. Description of the VZV DNA probe used for in situ hybridization. The viral DNA fragments (■), *Hind*III A (0.1 to 0.3 map units, 25.5 kilobases), B and D (0.39 to 0.65 map units, 16.5 and 12.9 kilobases), and C (0.85 to 0.95 map units, 12.9 kilobases) used to prepare the VZV DNA probe are shown in relation to the *Hind*III map of VZV DNA (19, 20) with gene regions as identified by Davison and Scott (9). The fragments were cloned in pBR322 by Richard Hyman, Pennsylvania State University, Hershey (6).

frequently by in situ hybridization than by viral culture, the pattern of clearance of VZV-infected PBMC was consistent with the isolation of VZV only from PBMC taken within 48 h (2, 4). The low frequency of positive cells accounted for

FIG. 2. Demonstration of VZV gene sequences in PBMC during primary VZV infection. PBMC obtained from subjects 24 h after the onset of acute varicella (A and B) and from a guinea pig 5 days after inoculation with 9×10^5 PFU of VZV (C) are shown after hybridization with the ^3H -labeled VZV DNA probe (magnification, $\times 63$); PBMC from one subject with varicella are shown after hybridization with the pBR322 vector DNA (D).

the observation that at least 2×10^5 PBMC had to be cultured to recover the virus (2). The concentration of silver grains over the nuclei of positive cells indicated that the presence of VZV in PBMC as previously detected by DNA-DNA hybridization was not attributable only to nonspecific uptake of viral particles (20, 21). Morphologically, PBMC that carried VZV gene sequences were lymphocytes. The fragility of PBMC from subjects with acute varicella led to significant destruction during fluorescence-activated cell sorting and produced a nonrepresentative cell sample, preventing the definitive association of VZV viremia with T or B lymphocytes. While the role of lymphocytes as one of the reservoirs for human cytomegalovirus (HCMV) has been demonstrated by in situ hybridization (18) and Epstein-Barr virus (EBV) is known to persist in B lymphocytes, these experiments proved that VZV infection of lymphocytes was a transient event during primary infection of the natural host. Viral gene sequences have also been detected in PBMC from some patients with herpes zoster, suggesting that these cells sometimes become infected during VZV reactivation (20).

Cell-associated VZV viremia was observed in strain 2 guinea pigs after subcutaneous inoculation with VZV adapted to replicate in guinea pig fibroblasts (3). When PBMC were tested at 2-day intervals, beginning 3 days after inoculation and continuing for 2 weeks, positive in situ hybridization was found only at 3 and 5 days after infection (Fig. 2). The frequency of VZV-infected PBMC was 0.001 to 0.002%. VZV was not detected by in situ hybridization in guinea pig splenocytes with the exception of one experiment in which cyclosporin A, 10 mg/kg for 5 days, was used to potentiate VZV infection. PBMC and splenocytes from mock-infected strain 2 guinea pigs were negative.

Because of homologies between gene sequences of the human herpesviruses, the specificity of VZV DNA probe was also evaluated in cells infected in vitro with human HCMV or herpes simplex virus 1 (Fig. 3) or transformed with EBV. Human foreskin fibroblast or melanoma cells inoculated with VZV (strain Chase) had >20 grains per cell over the nuclei in 75 to 80% of cells by in situ hybridization with the ^3H -labeled VZV DNA probe when 80 to 90% of cells showed cytopathic effect. When the VZV DNA probe was tested with cells infected with HCMV or herpes simplex virus 1 that exhibited an equivalent cytopathic effect, the maximum number of grains per cell did not exceed the low background observed with the pBR322 DNA probe (Fig. 3). B lymphocytes transformed with EBV demonstrated no

FIG. 3. Specificity of in situ hybridization of the VZV DNA probe with VZV gene sequences. VZV-infected melanoma cells inoculated with VZV (strain Chase) (0.5 PFU per cell) were examined by in situ hybridization with the ^3H -labeled VZV DNA probe (A) or with the pBR322 DNA alone (B) when 80 to 90% of cells showed cytopathic effect. Human foreskin fibroblast infected with HCMV (strain Hilton) (C) or Vero cells infected with herpes simplex virus 1 (strain MacIntyre) (D) were tested at an equivalent phase of cytopathic effect.

hybridization with the VZV DNA probe. In situ hybridization experiments with guinea pig embryo fibroblasts infected with guinea pig CMV (ATCC VR-682) and guinea pig herpes-like virus (ATCC VR-543) showed no increase in the number of grains over background (data not shown).

The lymphotropic potential of herpesviruses is difficult to investigate in vitro because low concentrations of virus are present and only a small percentage of cells are infected (18); it is further complicated in the case of VZV by the inability to achieve synchronous infection. In situ hybridization was used to examine the interaction of VZV with human B and T lymphocytes. B lymphocytes depleted of T cells were transformed with EBV (21) or stimulated with pokeweed mitogen (1 $\mu\text{g}/\text{ml}$), and T-lymphocyte cultures were stimulated with phytohemagglutinin (5 $\mu\text{g}/\text{ml}$). B- and T-lymphocyte cultures were infected with an ultracentrifuged preparation of sonicated VZV-infected melanoma cells at 1.5×10^4 PFU/ 10^5

cells. After 2 to 4 h, the cells were washed and suspended in RPMI 1640 with 15% fetal calf serum, 10% interleukin-2, and mitogen. Mitogen stimulation, which enhanced lymphocyte infection with other viruses (6, 7, 11, 13), allowed the maintenance of lymphocyte cultures long enough to follow the relatively prolonged course of VZV replication and reproduced the cell activation present during primary VZV infection in vivo. VZV replication was not detectable in mitogen-stimulated B-lymphocyte cultures but was significant after EBV transformation of B lymphocytes, with concentrations of >20 grains over the nuclei in >50% of cells tested 3 days after superinfection with VZV (Fig. 4). Cell membrane expression of VZV proteins was demonstrated in 60 to 75% of these cells by immunofluorescence with a fluorescein-isothiocyanate-conjugated monkey polyclonal antibody to VZV and with monoclonal antibodies to VZV gpI and p170 (1). Infectious virus was recovered (1.8×10^4 to 5.0×10^4 PFU/ml) for more than 14 days. In T-lymphocyte cultures, viral entry was evident with cytoplasmic grains visible in >75% of cells at 5 days after inoculation. Grains were distributed primarily just within the cytoplasmic membrane in most cells, while only 0.01% of T lymphocytes had >20 grains over the nucleus (Fig. 4). The breakdown of viral DNA in the cytoplasm by cellular DNases was demonstrated

FIG. 4. Demonstration of VZV gene sequences in PBMC infected with VZV in vitro. In situ hybridization with the VZV DNA probe is shown for EBV-transformed B lymphocytes at 3 days (A) and for phytohemagglutinin-stimulated T lymphocytes at 5 days (B and C) after VZV infection in vitro.

by 10 days, when only 25 to 40% of the cells had cytoplasmic grains. Nevertheless, >20 nuclear grains remained detectable in 0.01% of cells. Infectious VZV was isolated from phytohemagglutinin-stimulated T lymphocytes at 5 and 10 days, which is well beyond the survival time of the inoculum virus. Since this DNA virus must replicate in the nucleus, the few cells that demonstrated nuclear grains were considered productively infected. These experiments proved that VZV DNA reached the nucleus and replicated in a small percentage of T lymphocytes, as was consistent with the observation that VZV viremia in vivo was due to VZV infection of a few lymphocytes. Infection of human T lymphocytes with herpes simplex virus 1 and HCMV in vitro also showed that a small subset of cells supported virus growth (6, 7, 17). As was true of HCMV, most in vitro infection of lymphocytes was abortive (17) and, like adenovirus, the limited replication in human lymphocytes was not caused by impaired viral entry (11). The nonpermissiveness of most lymphocytes for VZV, despite the uptake of virion DNA, obviously benefits the host during primary infection, but the mechanism of the restriction has not been explained. One hypothesis, suggested by recent investigations of HSV cellular pathogenesis (8), is that adsorbed VZV virions entered most lymphocytes by endocytosis by using a pathway which did not involve fusion of the viral envelope and the plasma membrane of the cell and produced abortive infection.

The observation that activated T lymphocytes were permissive at low frequency for VZV infection in vitro suggested that lymphocyte activation, which alters cell surface antigens and was observed during the host response to primary VZV infection (2), could potentiate viremia. However, most PBMC remained nonpermissive in vitro and in vivo, and cells that harbored viral gene sequences in vivo were cleared rapidly by the immunocompetent host.

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