spread by this means throughout the country. However, modified-live vaccine viruses that we have tested had the original antigenic type (*11*), and the isolation of the new antigenic type of CPV from dogs in Europe (*12*) suggests that more than a simple vaccine spread was involved.

A third possibility is that the new virus is better adapted for replication in dogs, or that it spreads more efficiently. The surface antigenic structures of the viruses appear to be important in determining the host ranges of CPV or FPV in cats and dogs (*8*). It is possible that the original CPV was the first virus infecting dogs, and the virus has since evolved to better fit its new host. The loss of a CPV-FPV type-common epitope from the early isolates and the acquisition of a new CPV type-specific epitope by the new strain suggests that CPV may be evolving away from FPV to a form better adapted to dogs.

Small antigenic changes detectable by mAb's are common among viruses (*13*). However, the emergence of a new antigenic type only 2 or 3 years after the first recognition of CPV and the apparent replacement of the original virus by the variant are unusual. Further studies of this phenomenon will allow exploration of the factors important in the evolution of parvoviruses. As a practical matter these results suggest that it is unwise to regard parvoviruses as genetically invariant. For example, field CPV strains are now antigenically different from most vaccine strains in current use, and diagnostic tests that depend on mAb reactivities (*14*) could be invalidated by small antigenic changes in the virus.

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Detection of Human Cytomegalovirus in Peripheral Blood Lymphocytes in a Natural Infection

Abstract. *In situ hybridization was used to detect human cytomegalovirus (HCMV) in the peripheral blood mononuclear cells of some naturally infected (seropositive) individuals. A subpopulation of cells hybridized specifically to a portion of the HCMV genome that is heavily transcribed during the immediate-early period of infection. The hybridization signal was markedly reduced by base hydrolysis and ribonuclease, and therefore the probe appears to be detecting viral RNA. A fluorescence-activated cell sorter was used to select lymphocytes bearing the OKT4 and OKT8 markers. Hybridization with the HCMV probe revealed a higher proportion of positive cells in the OKT4 than in the OKT8 subset. This observation specifically identifies lymphocytes as a cell population involved in natural HCMV infection and suggests that lymphocytes may be a reservoir for maintaining infection and may also serve as a vehicle for its spread by blood transfusion.*

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A characteristic of the members of the herpesvirus family is their ability to remain in host tissue for many years after the initial infection and then to be reactivated, occasionally causing disease. Herpes simplex 1 and 2 and varicella zoster virus are maintained in sensory ganglia (*1*), and Epstein-Barr virus is maintained in B lymphocytes (*2*).

Human cytomegalovirus (HCMV) also establishes a latent infection, frequently after an asymptomatic infection in a healthy individual. It can be reactivated, usually in association with immunosuppression and transplantation surgery, to cause interstitial pneumonia and systemic disease (*3–6*). The site of latency and the molecular mechanisms of the establishment and maintenance of HCMV have yet to be determined. There is evidence that lymphocytes may play a role in HCMV infection. Individuals with HCMV mononucleosis have atypical lymphocytes, and in vitro tests reveal depression of lymphocyte functions (*7–9*). Approximately 70 percent of the adult population have antibodies to HCMV, indicating previous exposure, and seronegative individuals have been infected via blood transfusions from healthy seropositive donors (*10*). Depletion of leukocytes from seropositive blood decreases the incidence of transfusion-mediated transfer of HCMV infection, and some evidence points to the involvement of polymorphonuclear leukocytes (*11*). However, although HCMV can be isolated from the buffy coat of acutely infected patients (*7, 9–11*), attempts to detect HCMV infection in peripheral blood mononuclear cells of asymptomatic individuals have seldom been successful (*12*).

We (*13*) and others (*14*) independently found that a small percentage (average, 2 to 3 percent; range, 1 to 15 percent) of lymphocytes could be infected in vitro, as judged by expression of immediate-early (IE) and early HCMV proteins detected by monoclonal and monospecific antibodies. Past attempts to define the infection of lymphocytes with HCMV have been hampered by several factors including (i) the use of laboratory strains of HCMV that appear to be less lymphoinfectious than recent HCMV clinical isolates (*13*), (ii) the cryptic nature of the infection (*15*), and (iii) the difficulty of detecting low levels of virus in a small percentage of cells.

Our strategy for detecting viral nucleic acid in lymphocytes was to select as probes cloned viral fragments from regions of the HCMV genome that might be heavily transcribed in these cells. The RNA transcripts produced during the IE stage of HCMV infection have been mapped, characterized, and cloned (*16*).

The knowledge that nonpermissively infected cells often express early viral genes but are blocked in the transcription of late genes (*13, 14, 17*) led us to select the Eco RI J fragment as our probe (Fig. 1). This segment of HCMV DNA contains the coding sequence for the 1.9-kb major IE RNA, the most abundant transcript in the IE region, as well as the sequences for the 2.3 kb, 4.0 kb, and 7.0 kb IE transcripts (*16*). In previous experiments to detect HCMV antigen in peripheral blood mononuclear cells (PBM's) infected in vitro, we found that only a small proportion of cells were susceptible to infection (*13*). Therefore in situ hybridization appeared to be the technique most likely to detect HCMV RNA in these cells. Examination of PBM's from eight individuals who were seropositive for HCMV and twelve who were seronegative gave the following results. Strong hybridization with the pUC Eco RI J probe was found in 2 percent of the PBM's (dots containing 10^4 cells were scanned) from two of the eight asymptomatic seropositive individuals (Fig. 2, a and e). This level of hybridization was consistent over several months in six to eight different samples for each individual. Neither the vector plasmid probes pUC 18 or 19 (Fig. 2h), nor an HSV-2 probe (Bgl II fragment C) bound to these cells. Hybridization with the Eco RI J probe was found in 0.03 to 1 percent of the PBM's from the other six seropositive individuals. Cells from 11 of the 12 seronegative individuals showed no hybridization to the HCMV probe, but 0.1 percent of the cells from the 12th individual hybridized to the probe. The antibody may have been absent in this individual because viral protein was insufficient to generate a response or because the individual did not respond to this virus. A similar lack of detectable antibody has been observed in a subset of patients infected with human T-cell lymphotropic virus, type III (as determined by culture of virus) (*18*). Treatment with 0.1*M* NaOH (Fig. 2c) or ribonuclease (100 μg/ml, 30 minutes, 37°C) significantly reduced signal and indicated that hybridization was primarily with HCMV RNA. Heat treatment (100°C for 2 minutes) did not increase the hybridization signal in PBM's. In contrast, similar treatment of acutely infected fibroblasts increased the signal, presumably by hybridization of the probe to denatured viral DNA. Culture of lymphocytes from hybridization-positive donors on permissive fibroblasts produced no cytopathic effects; these results are consistent with many observations that HCMV-infected lymphocytes produce few, if any, infectious virions under such circumstances.

To determine if a specific subpopulation of PBM's harbored HCMV, we used a fluorescence-activated cell sorter (FACS) to separate cells from a seropositive (2 percent PBM-positive) individual. The lymphocytes were stained with antibodies detecting the two major T-cell subpopulations, OKT4 and OKT8. Although this technique provides few cells, the purity of the populations exceeds 97 percent. Our results from three such experiments indicated that a higher percentage of HCMV-hybridizing cells bore the OKT4 marker (2.4 percent) than the OKT8 antigen (0.8 percent) (Fig. 2, f and g) (in unseparated blood, 44 percent of the cells were OKT4 and 18 percent were OKT8). This observation not only demonstrates some selectivity of HCMV infection, but confirms that lymphocytes, specifically in the PBM preparations are infected and contain HCMV RNA. Whether HCMV RNA is present in other PBM subpopulations remains to be determined. Preliminary results indicate that monocytes also express the IE HCMV transcript.

The finding that HCMV RNA can be detected in circulating mononuclear cells harvested from some clinically healthy individuals has several implications. First, a population of cells capable of harboring HCMV has been identified. The presence primarily of HCMV RNA indicates that virus replication is restricted. Whether lymphocytes serve as the major reservoir for this virus or HCMV is maintained elsewhere and is seeded either constantly or periodically into lymphocytes as they traffic is not yet clear. It will be of interest to determine whether the viral DNA is integrated into the host genome or exists in a linear or plasmid form in the cell. Lymphocytes can be examined for the presence of other (later stage) RNA's and the expression of viral proteins.

Biologically, it will be important to know whether infectious HCMV can be reactivated from a lymphoid population, and if so, by what mechanism. Transfusion-mediated transmission would suggest that this can occur. This possibility is also suggested by murine experiments in which (i) latent murine cytomegalovirus (MCMV) has been found in a small subset of lymphocytes and in macrophages (*19*); (ii) MCMV has been activated by allogenic coculture of tissues containing those cells, and (iii) enhanced shedding of MCMV has been observed with MCMV-infected mice given allogenic skin transfers (*20*). Detection of HCMV in blood lymphocytes may serve not only as a marker for disease but may also help in the understanding of pathogenesis. It will be crucial to determine whether the percentage of cells expressing HCMV IE RNA varies in our asymptomatic seropositive donors (a subclinical reactivation) or is constant in certain individuals, and whether such donors are more likely to transmit infection. Also, the percentage of infected cells, the

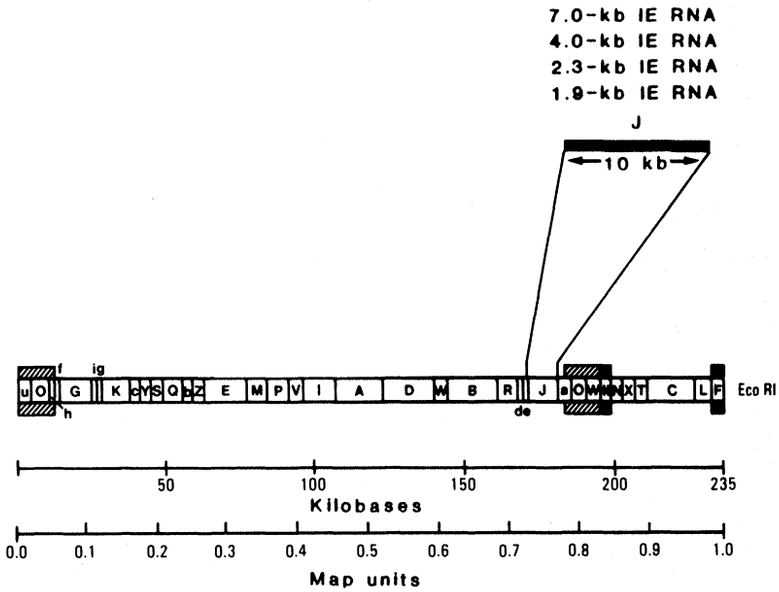

Fig. 1. The location of the IE viral probe utilized for in situ hybridization. A physical map for the Eco RI fragments of HCMV DNA, strain AD169, is shown with the relative position of the viral DNA fragment used for in situ hybridization. The known viral transcripts in these fragments are shown. Plasmids were prepared and the Eco RI J fragment was subcloned as described (*22*).

amount of HCMV genome expressed, and the type of lymphocyte infected may vary during clinical disease, possibly explaining the immunosuppression frequently accompanying clinical HCMV infection. As therapies for HCMV (such as antiviral drugs and cessation of immunosuppression after transplantation) are developed, rapid detection of clinical HCMV infections will be critical. Current methods in which dot-blot hybridization is used, indicate that HCMV-specific sequences increase significantly in blood before the onset of symptoms (*21*). The more sensitive in situ technique should permit detection of clinically dangerous levels of virus at an earlier stage, allowing rapid therapy.

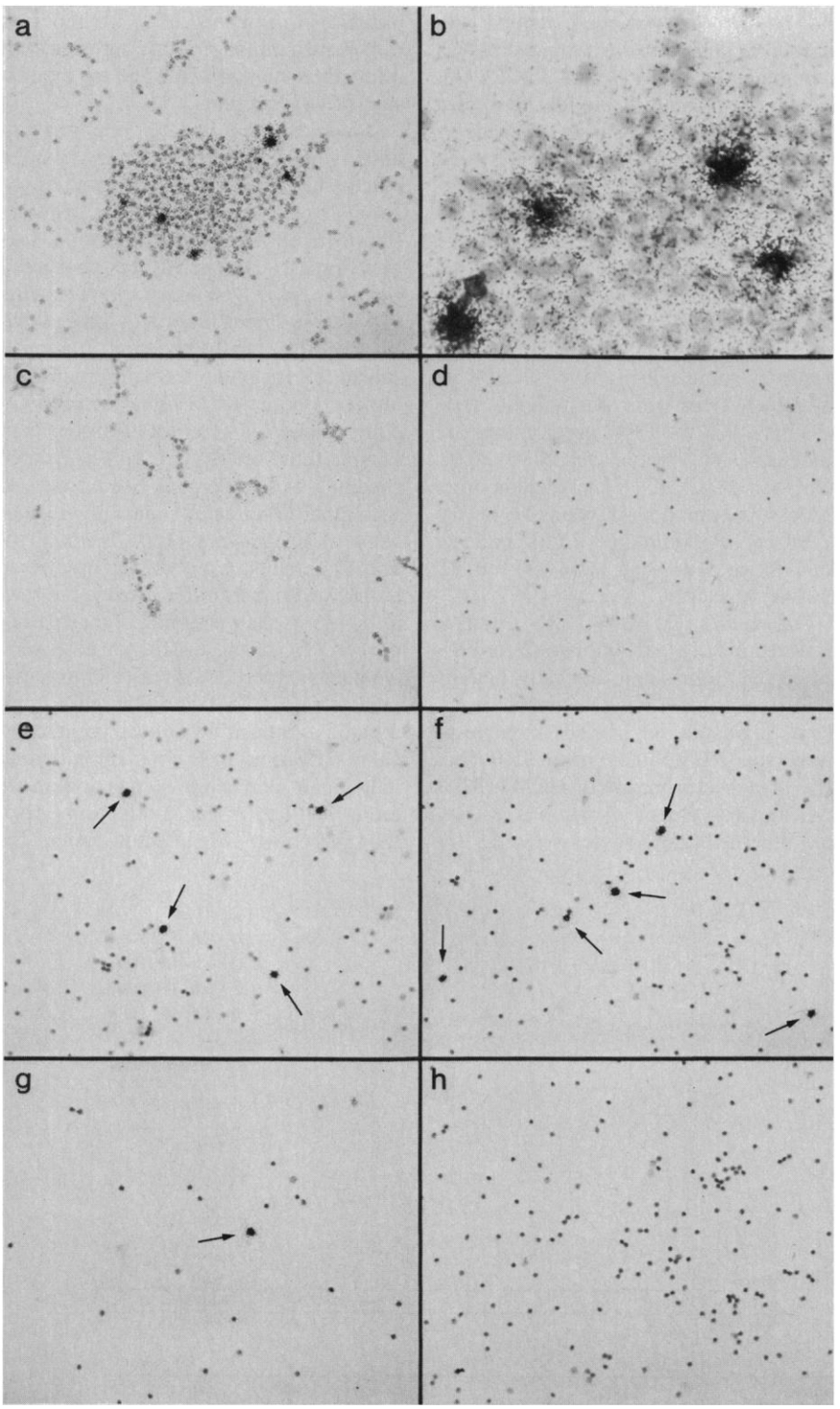

Fig. 2. (a) PBM's from HCMV-positive donor A hybridized (*23, 24*) with the ^{35}S-labeled probe pUC–Eco RI J at a magnification of ×20. (b) Same as (a) at a magnification of ×100. (c) PBM's from HCMV-positive donor A as hybridized with probe pUC–Eco RI J after treatment with 0.1*M* NaOH for 30 minutes at 37°C. (d) PBM's from a HCMV-seronegative donor hybridized with probe pUC–Eco RI J. (e) PBM's from HCMV-positive donor B hybridized with probe pUC–Eco RI J. (f) Cells from HCMV-positive donor B positively selected by FACS (*25*) for the OKT4 marker hybridized with probe pUC–Eco RI J. (g) Cells from HCMV-positive donor B positively selected by FACS for the OKT8 marker hybridized with ^{35}S-labeled pUC–Eco RI J. (h) PBM's from HCMV-positive donor B hybridized with ^{35}S-labeled pUC18.

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but not in uninfected or HSV-infected fibroblasts. Probes made from HSV genome (J. Nelson, unpublished results) hybridized to HSV-infected fibroblasts but did not bind HCMV-infected fibroblasts.

23. Seropositivity was determined by an enzyme-linked immunosorbent assay (ELISA), with desiccated infected fibroblasts used as the antigen (*30*). Serum that gave twice the response (as measured in optical density units) on HCMV-infected versus uninfected fibroblasts was defined as seropositive for CMV. T-cell proliferation to HCMV (2 background) after 5 days of culture correlated with results from the ELISA.
24. In situ hybridization was done according to modified methods of Brahic and Haase (*31*), Angerer (*32*), and Brigatti (*33*). Human peripheral blood mononuclear cells were obtained from normal seropositive and seronegative donors and isolated on Ficoll-Hypaque gradients. Cells were washed three times in phosphate-buffered saline (PBS), adjusted to 10×10^6 cell/ml and plated on glass slides coated with 0.01 percent polylysine. Air-dried cells on slides were fixed for 20 minutes with periodatelysine paraformaldehyde (PLP) (*34*) and for 15 minutes in 95 percent ethanol, and then were stored at 4°C. Briefly, fixed cells were hydrated through washing with graded ratios of ethanol to water, treated with 0.2*M* HCl (10 minutes), washed and permeabilized with 1 percent Triton X-100 for 1.5 minutes, postfixed with PLP for 4 minutes, washed in PBS-glycine (2 g/liter), then in PBS, and dehydrated to 100 percent ethanol. The hybridization mix consisted of 50 percent deionized formamide, 5× Denhardt's solution, 5× hybridization salts (0.9*M* NaCl, 50 m*M* NaH_2PO_4, and 5 m*M* EDTA), 10 percent Dextran sulfate, heparin (30 U/ml), salmon sperm DNA (500 μg/ml), and BHK cell RNA (250 μg/ml). ^{35}S-labeled probes were added to a concentration of 0.25 to 0.5 μg/ml (about 1×10^8 cpm/μg); the entire mixture was boiled for 2 minutes, then chilled on ice; and dithiothreitol was added to a concentration of 10 m*M*. The solution was placed on the cells, covered with sheets of Gel-bond (FMC Corporation, Rockland, Maine), and sealed with rubber cement. Hybridization was carried out for 18 hours at 37°C in a humidified chamber. Cover slips were then removed and slides were washed with 2× SSC (0.3*M* sodium chloride, 0.03*M* sodium citrate) for 30 minutes at 21°C, 30 minutes with 0.1× SSC at 21°C, 10 minutes with 0.1× SSC at 60°C, 20 minutes with 0.1× SSC at 37°C, and 5 minutes with 2× SSC, then dehydrated through ethanol and dried. Slides were dipped in Kodak NTB-2 emulsion, exposed for 3 to 4 days at 4°C, then developed with Kodak D19 and standard fixer.
25. Immunofluorescence for FACS sorting of T-cell populations was done as follows. Washed PBM's (2×10^7) were incubated for 40 minutes on ice in either biotinylated antibody to Leu3 (OKT4) or Leu2 (OKT8) diluted 1:10. After two washings in cold PBS, cells were incubated for 30 minutes on ice with fluorescein isothiocyanate-coupled Avidin at a dilution of 1:50 and washed and resuspended in 3 ml of PBS for sorting. All reagents were obtained from Beckton-Dickinson. Windows on the FACS (*35*) were set to select only for the very brightly staining cells.
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Kinetics of Energy Flow in the Phycobilisome Core

Abstract. *Light energy absorbed by the 576 bilin chromophores in the six rods of the phycobilisome of the cyanobacterium* Synechocystis *6701 is funneled into a 1.5 × 10^6 dalton core. The 72 bilins of the core function as a single unit with respect to the rate-limiting processes for energy flow within these particles.*

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In cyanobacteria and red algae, light spanning nearly the entire visible spectrum (450 to 650 nm) is used efficiently in photosynthesis. This light harvesting is performed by a family of intensely colored proteins, biliproteins, organized within macromolecular complexes called phycobilisomes (*1*). Detailed information is available on the structure of two cyanobacterial phycobilisomes (*2–5*). A schematic representation of the phycobilisome of *Synechocystis* 6701 is shown in Fig. 1. These phycobilisomes have molecular weights of $\sim 7 \times 10^6$ daltons and contain ~625 bilin chromophores. Energy absorbed by any one of these chromophores is transferred to the terminal acceptors within the phycobilisome (Fig. 1) with an efficiency >97 percent with respect to the fluorescence of isolated phycobiliproteins (*6*).

In an earlier study (*6*), we examined the kinetics of energy flow within the *Synechocystis* 6701 phycobilisome and showed that disk-to-disk transfer was the rate-limiting step for energy flow. Under the assumption that the phycoerythrin-to-phycoerythrin and phycoerythrin-to-phycocyanin transfer times are equal, the average disk-to-disk transfer time in wild-type phycobilisomes (Fig. 1A) was calculated to be 24 ± 4 psec (mean ± standard error of the mean). For 620-nm excitation of phycocyanin in mutant strain CM25 phycobilisomes (Fig. 1B), the rise time of terminal acceptor emission at 680 nm was 25 ± 4 psec (*6*). This rise time includes delays arising from disk-to-disk transfer from phycocyanin to phycocyanin and from phycocyanin to allophycocyanin, as well as from any energy transfer within the core. We predict on the basis of our previous results (*6*) that, if the phycocyanin-to-phycocyanin energy transfer time in the rods and the phycocyanin-to-allophycocyanin transfer time between the rods and the core are similar to the average phycoerythrin-to-phycoerythrin and phycoerythrin-to-phycocyanin disk-to-disk transfer time calculated in our previous study, then the transfer of energy from allophycocyanin to the terminal acceptors within the core must take place in <10 psec (*7*). This is a remarkably rapid transfer rate since the core contains 68 allophycocyanin chromophores and only four terminal acceptor chromophores, two on α^{APB} polypeptides and two on L_{CM}^{99} polypeptides (*3*).

We describe here experiments that test our prediction. This test was made possible by the isolation of highly purified phycobilisome cores (*8*) from a recently described mutant of *Synechocystis* 6701, strain UV16 (*5*). The purified cores were examined by electron microscopy and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), as well as by steady-state absorption and emission spectroscopy. Electron microscopy of the purified core preparation (Fig. 2) showed only particles virtually devoid of rod components with the morphology of typical *Synechocystis* 6701 wild-type phycobilisome cores (*4, 5, 9*).

A comparison of the polypeptide patterns of wild-type and strain CM25 phycobilisomes, and of purified cores of strain UV16, was obtained by means of SDS-PAGE. Densitometric quantitation of such gels showed that the purified cores contained all of the core polypeptides present in wild-type phycobilisomes, except that the level of L_{RC}^{27} (see Fig. 1A) was ~20 percent lower in the purified cores than in the wild-type or CM25 particles. Since a considerable portion of this polypeptide lies outside the core and is normally protected by the proximal phycocyanin, it is possible that the decrease in the amount of this polypeptide is due to proteolytic cleavage in strain UV16, either within the cells or during preparation.