Gliomas & CMV

Supporting papers
References


A-3  San Francisco Chronicle Oct6, 2008 "Surgeon changes study of brain tumours” by Justin Berton


A-9  International Herpes Management Forum 3rd Annual Meeting Nov 1995. The increasing importance of CMv, Epstein-Barr Virus and the Human Herpes viruses types 6,7,8


A-14  Enhanced Replication of Human Cytomegalovirus in Human Fibroblasts Treated with Dexamethasone by Junji Tanaka et al, Department of Virology, Cancer Research Institute, Kanazawa University, Kanazawa 920, Japan, 6-Jun-1984


A-16  Incidence of cytomegalovirus infection among the general population and pregnant women in the United States. Fernando AB Colugnati et al. BMC Infectious diseases 2007, 7:71
Brain cancer

- Brain cancer incidence and mortality rates are expected to remain unchanged at 2006 levels.

- Between 1997 and 2006, the incidence rates of brain cancer showed no significant trend in males and females for all ages combined but fell by 56.8 per cent in males aged 80 years and older at diagnosis.

- Incidence rates in people in NSW aged less than 50 years did not change in the most recent 10-year period.

- For the longer term, over 36 years, joinpoint regression analysis shows that the average annual increase in brain cancer incidence was 0.8 per cent per annum from 1975 to 2004. Rates have declined in males by eight per cent a year since 2004.

- In females, incidence rates increased by 3.0 per cent per annum until 1983, thereafter rates have remained constant.

In 2006, there were 464 new cases of brain cancer in NSW, 249 in males and 215 in females, or 1.3 per cent of all cancers in males and 1.4 per cent in females. Of the 319 cancer deaths from brain cancer in 2006, 192 were in males and 127 in females. These represented 2.6 per cent of male cancer deaths and 2.2 per cent of female cancer deaths.

After allowing for differences in age, males were 1.3 times more likely than females to be diagnosed with brain cancer and 1.7 times more likely to die from it.

Brain cancer incidence ranked 16th overall, 15th in males and 16th in females, and its mortality ranked 14th overall, 14th in males and 11th in females. Incidence rates were 7.3 new cases per 100,000 in males and 5.7 in females and mortality rates were 5.5 deaths per 100,000 in males and 3.3 in females.

The current likelihood or risk of developing brain cancer by the age of 75 years is one in 179 in males and one in 213 in females. The likelihood increases to one in 114 in males and one in 140 in females if the risk is considered to the age of 85 years.

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i. Unless otherwise stated all rates are standardised to the 2001 Australian population. Rates standardised to the Australian and World 1960 population are provided in tables. Rates standardised to the World population will be lower than the Australian population because the 1960 population has a greater proportion in younger age groups.
In both sexes brain cancer is the second most common cancer in children aged five to 14 years. With increasing age, incidence and mortality rates are twice as high in males as they are in females. In 1977, the median age at diagnosis of brain cancer was 49 in males and 56 in females, increasing to 59 in males and 63.5 in females in 2006. Similarly, the median age at death was 57.5 years in males and 59 in females in 1977, increasing to 62 years in males and 66 years in females in 2006. Increases in the median age at diagnosis and at death are as a result of the population ageing and a later age at diagnosis.

Numbers of new cases and deaths by age group

<table>
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<tr>
<th>Age (yr)</th>
<th>0 to 49</th>
<th>50 to 64</th>
<th>65 to 79</th>
<th>80+</th>
<th>Total</th>
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<tr>
<td>Incidence</td>
<td>141</td>
<td>155</td>
<td>150</td>
<td>58</td>
<td>564</td>
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<td>Per cent of total incidence</td>
<td>30.4%</td>
<td>24.8%</td>
<td>32.3%</td>
<td>12.5%</td>
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<td>Mortality</td>
<td>44</td>
<td>110</td>
<td>110</td>
<td>38</td>
<td>307</td>
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<tr>
<td>Per cent total deaths</td>
<td>14.3%</td>
<td>35.8%</td>
<td>35.8%</td>
<td>12.4%</td>
<td>100%</td>
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Projections of incidence and mortality

In 2008, the estimated number of new cases of brain cancer is expected to increase to 507: 294 in males and 213 in females. Incidence rates are estimated to decline to 8.2 new cases per 100,000 in males and remain at 2006 levels in females, with 5.5 new cases per 100,000.

The number of deaths in 2008 is expected to increase to 366 deaths overall: 217 cancer deaths in males and 149 in females. Mortality rates are expected to remain at 2006 levels in males and females with 6.0 and 3.7 deaths per 100,000 respectively. While the projected number of new cases and deaths based on historical trends provide the best estimates, they do assume that historical patterns will continue in the future.

Brain cancer

**Time trends – incidence by age category**

In 2006, there were 141 new cases of brain cancer diagnosed in people aged 0 to 49 years, or 30.4 per cent of total cases; 115, or 24.8 per cent, in people aged 50 to 64 years; 150, or 32.3 per cent, in people aged 65 to 79 years; and 58, or 12.5 per cent, in people aged 80 years or older. Unlike other cancers, the majority of brain cancers occur in younger age groups.

Brain cancer incidence rates were examined by age category at the time of diagnosis and then trends considered over time in males and females. In 2006, rates in males (cases per 100,000) were 3.3 in males aged 0 to 49, 11.4 in males 50 to 64 years, 24.3 for those aged 65 to 79 years and 23.7 for males aged 80 years and older. In females, the rates were 2.6, 7.5, 20.4 and 22.6 cases per 100,000 respectively.

The percentage change in incidence rates for brain cancer for the period 1997 to 2006 is presented overall and by age category. Only statistically significant changes are mentioned. Between 1997 and 2006, the incidence rates of brain cancer showed no significant trend in males and females for all ages combined and fell by 56.8 per cent in males aged 80 years and older. In females, the rates were 2.6, 7.5, 20.4 and 22.6 cases per 100,000 respectively.

The average increase in brain cancer incidence was 0.8 per cent per annum from 1975 to 2004. Rates have declined in males by 8.0 per cent a year since 2004; the reason for this decline is uncertain at this stage. In females, incidence rates increased by 3.0 per cent per annum until 1983. Thereafter rates have remained constant. Mortality rates increased from 1.7 per cent in males and 1.8 per cent in females up until 1994 and 1991 respectively. Thereafter rates declined in males and females by 1.3 per cent respectively.

**Time trends – mortality by age category**

In 2006, there were 44 deaths in people aged 0 to 49 years, or 14.3 per cent of total brain cancer deaths; 110, or 35.8 per cent, in people aged 50 to 64; 110, or 35.8 per cent, in people aged 65 to 79 years and 38, or 12.4 per cent of brain cancer deaths, in those aged 80 years and older.

Brain cancer mortality rates were examined by age at death and then trends considered over time. Brain cancer deaths per 100,000 were 1.4 in males and 1.0 in females aged 0 to 49 years; 11.9 in males and 4.4 in females aged 50 to 64 years; 20.0 in males and 11.7 in females aged 65 to 79; and 28.11 and 15.6 in males and females aged 80 years and older.

The percentage change in mortality rates for the period 1996 to 2007 is presented overall and by age category.

Mortality rates:

- fell by 15.3 per cent in males for all ages combined; and
- fell by 33.3 per cent in males aged 0 to 49 years and by 68.9 per cent in males aged 80 years and older.

**Time trends – over 36 years**

For the longer term, over 36 years, using joinpoint regression analysis the average increase in brain cancer incidence was 0.8 per cent per annum from 1975 to 2004. Rates have declined in males by 8.0 per cent a year since 2004; the reason for this decline is uncertain at this stage. In females, incidence rates increased by 3.0 per cent per annum until 1983. Thereafter rates have remained constant. Mortality rates increased from 1.7 per cent in males and 1.8 per cent in females up until 1994 and 1991 respectively. Thereafter rates declined in males and females by 1.3 per cent respectively.
Figure 19  Brain cancer

Age-standardised incidence, NSW

Age-standardised mortality, NSW

Age-standardised incidence by age category, NSW males

Age-standardised mortality by age category, NSW males
Brain cancer

**International variation**

From 1998 to 2002, NSW incidence rates for brain cancers, standardised to the ‘world’ population, were 6.6 in males and 4.6 in females per 100,000. Males in Croatia had the highest rates with 10.2 and females in Kielce, Poland, with 8.3 per 100,000. The lowest rates were in Osaka, Japan, with 2.5 in males and 2.0 in females.

For comparative purposes we selected regions that have similar reporting standards for cancer to NSW and that provide information on OECD health indicators and risk factors. The region with the highest rates in males was Ireland with 7.5 per 100,000 and Finland, with 5.2 per 100,000 in females (Figure 20).

In an earlier period, 1993 to 1997, NSW incidence rates for cancers of the brain and central nervous system were 6.9 in males and 5.0 per 100,000 in females. Rates were similar to Canada, Denmark, New Zealand and the USA for males and females. Rates were slightly higher than in the United Kingdom and Japan with 2.5 in males and 1.6 in females. The highest rates among the selected countries occurred in Denmark with 7.3 in males and 5.0 in females.

**Figure 20** Age-standardised incidence rates for brain cancer in males and females (1998–2002)
Survival and prevalence

The five-year relative survival experienced from 1999 to 2003 in NSW was 19 per cent for males and 21 per cent for females diagnosed with brain cancer. Five-year relative survival for brain cancer declined dramatically with age. For those aged 15 to 44 years at diagnosis, five-year survival was 55.3 per cent, declining to 7.7 per cent in those aged 55 to 64 and 4.4 per cent in those aged 75 years and older. Five-year survival from brain cancer was similar across all time periods from 19.8 per cent for cancers diagnosed in 1980–1983 to 20.0 per cent in 1984–1988, 19.7 per cent in 1989–1993, 17.0 per cent in 1994–1998 and finally to 20.4 per cent in the latest time period 1999–2003.iv

There were 1,625 people, 918 males and 707 females, living with brain cancer at the end of 2004 who were diagnosed between 1980 and 2004. Of these, 651 people, 373 males and 278 females, or 40.1 per cent had been diagnosed within five years prior to December 2004. Five-year prevalence rates (per 100,000) were 9 overall, 10 in males and 7 in females. For every person diagnosed with brain cancer in 2006, there was another male and female living with their brain cancer who had been diagnosed in the previous five years.

Risk factors for brain cancer

There are very few known risk factors for brain cancer. One established risk factor is the exposure to ionised radiation to the head, usually received for treatment of other cancers.32,33 Most commonly this is seen in leukaemia patients who received radiation therapy as part of their treatment. Children younger than 14 years of age appear to be at a higher risk for brain cancer than are older children.33

Some types of brain cancers have been suggested to be associated with exposure to certain chemicals used in oil refining, rubber manufacturing, vinyl chloride, petroleum, pesticides and nuclear industries.34 But, a definite link between exposure to these chemicals and brain tumours has not been proven.33,35 Similarly, there has been speculation that long-term use of cell phones increases the risk of brain cancer, but the evidence is conflicting on a population level.33,35

People with impaired immune systems, side effects from cancer treatment, immunosuppressive therapy or HIV infection are also at increased risk.33
Age and sex-specific tables by cancer type

Description of tables

The following tables, three for new cases and a corresponding three for deaths are grouped as follows:

Tables 18 to 21 – provide a breakdown of new cases and deaths for five-year age groups from 0–4 years to 95 years and older, the total numbers of cases and deaths and the percentage of all cases for the same sex.

Tables 19 and 22 – includes age specific incidence and mortality rates, the crude rate, cumulative rate to age 75, cumulative risk expressed as ‘one in’ to age 75 and 85 and rates directly standardised to the age distribution of the standard world population and the population of Australia in 2001. The crude and age-standardised rates are expressed per 100,000 populations, whereas the cumulative rate is a percentage.

Tables 20 and 23 – has two directly age-standardised rates with 95 per cent confidence intervals.

Grouping of cancer sites and deaths

All tables include results for the three groups of cancers: head and neck, colorectal (bowel) and all leukaemias. Note that ‘head and neck’ includes cancers of oral cavity (mouth (C01-C06), salivary glands (C07-C08), pharynx (C09-C14)) and upper respiratory tract (nose, sinuses, etc (C30-C31) and larynx (C32)). It does not include lip cancer (C00).

The total number of cancers is referred to as ‘All sites except non-melanocytic skin cancers’. As well as melanocytic skin cancers, the total includes Kaposi’s sarcoma of the skin and cutaneous lymphomas. Other rare non-melanocytic skin cancers such as Merkel cell tumours are tabulated by site. Basal and squamous cell carcinomas of the skin are not registered and not included.

New category

There is a new category of myelodysplasia and myeloproliferative disorders. Historically, these have been considered pre-leukaemic and not notified to the registry. Since the introduction of ICD-03 these conditions are now considered invasive.
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Human Cytomegalovirus Infection and Expression in Human Malignant Glioma

Charles S. Cobbs, Lualhati Harkins, Minu Samanta, G. Yancey Gillespie, Suman Bharara, Peter H. King, L. Burt Nabors, C. Glenn Cobbs, and William J. Britt

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Abstract

Malignant gliomas are the most common primary brain tumors in adults, have no known etiology, and are generally rapidly fatal despite current therapies. Human cytomegalovirus (HCMV) is a β-herpesvirus tropic for glial cells that persistently infects 50–90% of the adult human population. HCMV can be reactivated under conditions of inflammation and immunosuppression, and HCMV gene products can dysregulate multiple cellular pathways involved in oncogenesis. Here we show that a high percentage of malignant gliomas are infected by HCMV and multiple HCMV gene products are expressed in these tumors. These data are the first to show an association between HCMV and malignant gliomas and suggest that HCMV may play an active role in glioma pathogenesis.

Introduction

Human herpesviruses are implicated in the pathogenesis of several human malignancies (1). HCMV is a β-herpesvirus endemic in the human population that can cause devastating encephalitis in fetuses and immunocompromised adults (2). HCMV gene transcription can be activated in astrocytic cells by inflammatory stimuli, and transcriptionally active HCMV can induce malignant transformation and dysregulate key cellular pathways involved in mutagenesis, cell cycle, apoptosis, angiogenesis, cell invasion, and host immune response (3–13). Malignant gliomas are thought to be primarily astrocytic in origin and are characterized by increasing degrees of DNA instability, cell proliferation, angiogenesis, and microscopic invasion, with the most malignant form being WHO grade IV astrocytoma (also termed glioblastoma multiforme or GBM; Ref. 14). We decided to investigate whether HCMV might be involved in glioma pathogenesis because several important cellular pathways in glioma biology that we study also promote, and are promoted by, HCMV gene expression.

Materials and Methods

IHC. Human glioma and meningioma surgical specimens, and Alzheimer’s disease, stroke, encephalitis, cerebritis, and normal brain autopsy specimens were obtained in paraffin blocks (with Institutional Review Board approval) and sectioned (6 μm). Sections were blocked for endogenous peroxidase (3% H₂O₂, for 12 min) and incubated with Fc receptor blocker (10 min, at 20°C; Innovex Biosciences, Richmond, CA) before the addition of an mAb. We performed IHC using three different three-stage horseradish peroxidase detection systems (BioGenex, San Ramon, CA; Dako, Carpinteria, CA; and Innovex Biosciences) with the following mAbs: anti-IE1–72 (1:25; BioGenex), anti-pp65 (1:30; Novocastra, Newcastle upon Tyne, United Kingdom), anti-p52/76kD (1:30; Novocastra), and anti-CD34 (1:15; BioGenex). Antibody parameters (e.g., postfixation, retrieval, and incubation time) were established for each mAb using three different three-stage horseradish peroxidase detection systems (BioGenex) with negative (nonspecific DNA) and positive (specific for polyadenylic mRNA) controls (HSV-1/2) probes were obtained (ResGen/Invitrogen, Huntsville, AL). We performed enzyme digestion and nuclear acid denaturation of paraffin sections using a Misha thermocycler (Shandon Lipshaw, Pittsburgh, PA), and slides were hybridized overnight at 37°C in a humidified chamber (methods are detailed in manuscript in preparation).4 Probe was detected using a supersensitive detection system (BioGenex, chromogen NBT). To detect HCMV DNA, we used a digoxigenin-labeled HCMV total genome DNA probe (Zymed Labs, South San Francisco, CA). Positive (specific for endogenous alu DNA sequence) and negative (nonspecific DNA) digoxigenin-labeled control probes were provided by the manufacturer.

Double Labeling of Paraffin Sections for HVMC DNA and GFAP Proein.ISH for HCMV DNA using the digoxigenin-labeled probe was performed as described. Citra antigen retrieval (BioGenex), endogenous peroxidase block (3% H₂O₂, 12 min), and Fc receptor block treatments and then anti-GFAP polyclonal antibody were applied (Zymed Labs, 2 h, 20°C). GFAP was detected with a multilink alkaline phosphatase detection system (BioGenex) and NBT chromogen.

Nested PCR and DNA Sequencing. Glioma DNA was purified from paraffin-embedded surgical specimens of malignant gliomas in accordance with the Institutional Review Board using DNeasy Tissue System (Qiagen, Valencia, CA) according to the manufacturer’s instructions. To avoid contamination, no positive controls were used for PCR, and PCR was carried out using unlinked primer-specific primers specific for HCMV glycoprotein B (UL53) gene as described with minor modifications (15). Amplified DNA products from tumors were visualized on agarose gels with ethidium bromide, bands were cut out, and DNA was extracted and analyzed by automated sequencing (ABI Model 377 DNA Sequencer). Confirmation of HCMV sequences was performed using a National Center for Biotechnology Information BLAST search. DNA extractions, PCR amplifications and DNA sequencing were repeated on several tumors in a blinded fashion to confirm these findings.

EM-IHC. GBM tissues were obtained at surgery and prepared for EM-IHC with anti-pp65 Ab (Novocastra) as described previously (16). Secondary antibodies bound to 35-nm gold particles (Electron Microscopy Sciences, Fort Washington, PA) were used for visualization.

Results

To determine whether HCMV was present in malignant gliomas, we performed IHC on paraffin sections from 27 malignant glioma surgical specimens obtained from nonimmunocompromised patients using a mAb specific for the HCMV-encoded IE1–72 protein. After...
optimizing conditions for low levels of expression, we could detect IE1–72 immunoreactivity in 27 of 27 malignant glioma biopsy specimens of various grades (WHO grades II-IV) but not in meningioma biopsy specimens or autopsy specimens from patients with Alzheimer’s disease, stroke, or encephalitis, or from patients without CNS disease (Fig. 1; Table 1). In GBMs, IE1–72 immunoreactivity was localized in both the perinuclear cytoplasm and the nucleus of tumor cells. In general, blood vessels within tumors, areas of necrosis, and areas of adjacent normal-appearing brain were minimally or not at all immunoreactive (Fig. 1). No immunoreactivity of tumor cells was observed in malignant gliomas when primary antibody was excluded, or when an IgG1 isotype-identical anti-CD34 mAb (specific for endothelial cells) was used. To further assess the extent of HCMV protein expression, we performed IHC on a subset of these glioma specimens using mAbs specific for: (a) HCMV pp65 tegument protein and (b) HCMV M, 76,000 early protein and M, 52,000 delayed-early DNA binding protein. Immunoreactivity was detected in at least some tumor cells in all of the glioma specimens that were examined but not in control brain using these mAbs (Fig. 1; Table 1).

To confirm that HCMV nucleic acids were present in the same cellular distribution as HCMV proteins in these tumors, we performed ISH to detect HCMV nucleic acids in gliomas and controls using two different HCMV probes and detection systems. Using a biotinylated 21-base oligonucleotide probe specific for HCMV immediate-early gene mRNA, we detected HCMV nucleic acids in glioma specimens but not in controls (Fig. 2, A and D; Table 2). No signal was detected in gliomas when a biotinylated 21-base oligonucleotide probe with similar GC content specific for HSV-1 and -2 (HSV-1/2) was used, or when the probe was eliminated from the hybridization reaction (Fig. 2, B and E). A positive control probe (specific for polyadenylated mRNA) hybridized with all of the specimens analyzed (gliomas and controls; Fig. 2, C and F). We repeated these experiments using a digoxigenin-labeled probe specific for DNA from the entire HCMV genome (Fig. 2, G–N; Table 2). A nonspecific DNA digoxigenin-labeled probe (Fig. 2, H and K), and a digoxigenin-labeled probe specific for endogenous alu DNA sequences (Fig. 2, I, L, and N) were used as negative and positive controls, respectively. With both of the ISH techniques, we detected HCMV nucleic acids in glioma tumor cells, in endothelial cells in the tumors, and occasionally in tumor-associated vascular smooth muscle cells, but not in normal brain controls (Fig. 2M) nor in areas of normal brain adjacent to tumor. We confirmed that HCMV DNA was present in cells of glial origin by performing double-labeling experiments using the HCMV DNA ISH probe, followed by IHC with an anti-GFAP antibody (Fig. 2O). In glioma specimens that invaded normal brain, we found that HCMV nucleic acids and IE1–72

Table 1 Detection of HCMV proteins in different-grade gliomas, meningiomas, other CNS diseases, and normal brain

Results from IHC with antibodies to four HCMV proteins and CD34 are shown. Anti-IE1-72 antibody was reactive with HCMV-positive control tissues and nonreactive with HSV-1-infected tissue (not shown).

<table>
<thead>
<tr>
<th>Specimen</th>
<th>IE1-72</th>
<th>pp65</th>
<th>p52/76kD IE/EA</th>
<th>CD34*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioblastoma</td>
<td>22/22</td>
<td>8/8</td>
<td>8/8</td>
<td>0/22</td>
</tr>
<tr>
<td>Grade III oligoastrocytoma</td>
<td>1/1</td>
<td></td>
<td></td>
<td>0/1</td>
</tr>
<tr>
<td>Grade II astrocytoma</td>
<td>4/4</td>
<td>2/2</td>
<td>2/2</td>
<td>0/4</td>
</tr>
<tr>
<td>Normal brain</td>
<td>0/5</td>
<td>0/1</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Meningioma</td>
<td>0/9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroke</td>
<td>0/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>0/3</td>
<td></td>
<td></td>
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<tr>
<td>Paraneoplastic encephalitis</td>
<td>0/1</td>
<td></td>
<td></td>
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<tr>
<td>Cryptococcal cerebritis</td>
<td>0/1</td>
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</table>

*Immunoreactivity was present in blood vessels only.
protein expression were readily detected in areas of tumor but were not detected in areas of normal-appearing brain within the same section (Fig. 1, C and D). To confirm the specificity of our HCMV oligonucleotide ISH probe and anti-IE1–72 mAb in gliomas, we infected human U251 GBM cells in culture with HCMV strain AD169 (multiplicity of infection, 5–10) and performed ISH and IHC. By both IHC and ISH, we could readily detect IE1–72 immunoreactivity and HCMV nucleic acid hybridization, respectively, in AD169-infected U251 cells but not in uninfected U251 cells (not shown).

To further analyze the HCMV present in these glioma specimens, we selected cases that were positive for HCMV by ISH and extracted DNA from the original paraffin blocks for use in PCR reactions to amplify HCMV nucleic acids. Using DNA from paraffin sections of nine different malignant gliomas, we performed nested PCR for a polymorphic region of the HCMV glycoprotein B (UL55) gene. We amplified UL55 in seven of nine of these paraffin-derived GBM DNA samples with nested PCR.

A 141-bp PCR product was cut from the agarose gels of these PCR reactions, the DNA was extracted, and direct DNA sequencing was performed. Of these seven tumors from which UL55 DNA sequencing was performed, two tumors had a gB-1 (Towne prototype) genotype, three tumors had a gB-2 (AD-169 prototype) genotype, and two tumors had a variation of either genotype (data not shown).

To determine whether HCMV viral particles could be identified in human gliomas, two GBMs obtained at the time of surgery were

<table>
<thead>
<tr>
<th>Biotinylated oligo probe specific for HCMV RNA</th>
<th>HSV-1/2</th>
<th>HCMV digoxigenin-labeled total genome DNA probe for HCMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioblastoma</td>
<td>8/8</td>
<td>4/4</td>
</tr>
<tr>
<td>Grade II astrocytoma</td>
<td>2/2</td>
<td>2/2</td>
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<tr>
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analyzed by immunogold EM-IHC. We used an anti-pp65 mAb labeled with a secondary antibody bound to gold particles. In both tumors, rare electron-dense particles labeled with gold were identified that were morphologically consistent with HCMV virions (Fig. 3).

Discussion

These data are the first to show that HCMV nucleic acids and proteins are present in a high percentage of low- and high-grade malignant gliomas, and that the expression of early and delayed HCMV gene products occurs in these tumors. Although these data do not establish a causal role for HCMV in glioma pathogenesis, a wealth of existing data indicates that HCMV could facilitate glioma progression. Human herpesviruses are implicated in the pathogenesis of several human cancers, although the exact mechanisms of oncogenesis are incompletely understood. In EBV- and human herpesvirus 8-related malignancies, viral reactivation is believed to occur after years of latency and to lead to malignancy (1). Factors such as inflammatory cytokine activation of latently infected cells and inborn and/or acquired host immunological characteristics are thought to be important in determining which individuals with these viral infections succumb to malignancies. We hypothesize that HCMV infection of glioma cells may be attributable to reactivation of an underlying persistent astrocytic or endothelial cell infection, or to de novo infection of glial cells that have acquired defects in cell cycle control mechanisms. HCMV gene expression in a glial cell that does not lead to cell cycle arrest or apoptosis might promote clonal expansion without producing a productive or cytopathic viral infection. Indeed, existing data indicate that long-term passage of HCMV in malignant glioma cells can result in the occurrence of variant strains with minimal cytopathic effect, and that HCMV can be reactivated in latently infected glioma cells when the cells are exposed to inflammatory stimuli or superinfected with other HCMV strains (17, 18). Sustained expression of specific HCMV gene products in such a setting might promote the overall glioma phenotype because HCMV encodes for gene products that can dysregulate cellular pathways involved in mutagenesis, apoptosis, cell cycle, angiogenesis, cell invasion, and host antitumor immune response (5–13). HCMV gene products can also transactivate other oncogenic viruses that are associated with malignant gliomas such as JC virus (19, 20), and may synergize with such viruses to promote oncogenesis. Thus, the potential role or roles of HCMV in glioma pathogenesis may not conform to existing paradigms in viral oncogenesis, and further research based on these findings may lead to a better understanding of the biology of this malignancy and reveal novel treatment or prevention strategies.
Surgeon changes study of brain tumors
Justin Berton, Chronicle Staff Writer
Monday, October 6, 2008

(10-05) 17:34 PDT -- As a young neurosurgery resident at UCSF in the late '90s, Dr. Charles Cobbs developed a hunch about brain tumors. It was a theory that he now concedes "was not based on a lot of scientific things."

Cobbs had observed that his patients diagnosed with malignant glioma - an aggressive brain cancer that leaves victims with a two-year life expectancy - were mostly older, well-educated and from higher socioeconomic backgrounds.

Their "hyper-hygienic" lifestyles had possibly left their immune systems susceptible to more common viruses, such as the human cytomegalovirus, or CMV, a herpes virus so ubiquitous that it infects 4 of 5 Americans.

During off-hours, and without formal research funding, Cobbs and a lab partner analyzed dozens of brain tumor samples: All of them were riddled with CMV.

In 2002, the doctor published his novel finding in a leading medical journal Cancer Research where it was quickly dismissed by many of his peers.

"I was left with a lot of self doubt," said Cobbs, now 45. "My fear was that we'd done something incorrect. But now, my confidence is growing."

In February, brain cancer researchers at Duke University Medical Center published the first peer-reviewed report that confirmed Cobbs' discovery, followed by two reports from independent labs at the M.D. Anderson Cancer Center at University of Texas in Houston and the Karolinska Institute in Stockholm, Sweden. And this month, the National Brain Tumor Society is sponsoring a first-of-its-kind gathering in Boston of the world's top virologists and glioma experts to examine the possible link between CMV and the deadly brain tumors that are diagnosed in 10,000 Americans every year.

"His discovery opens the door and has broad implications in this field," said Dr. Duane Mitchell, a Duke University Medical Center researcher who is conducting vaccine trials based on Cobb's findings. "And the door has just been opened."

An unorthodox connection

Cobbs came up with the idea to connect CMV and brain tumors while reading "Surely, You're Joking Mr. Feynman!" a collection of reminiscences on the value of original thinking by Nobel Prize-winning physicist Richard Feynman. The book inspired Cobbs to re-examine long-held assumptions in his field.

Medical researchers have long known that CMV exists in a latent state for most people unless a
person's immune system is compromised. While it's a common and unremarkable virus in some ways, CMV also causes persistent infection and is known to carry cancer-causing properties. It's also the most common cause of congenital brain infections in humans, according to the Centers for Disease Control and Prevention.

Cobbs expected another researcher had considered CMV as a likely culprit for brain tumors, but he found no published evidence.

"When I stopped to think, 'If I was going to cause a brain tumor, what would I be?" Cobbs said, "CMV made a lot of sense. ... But if you tell people you think like that, in these days of rigid grant proposals - well, it might not win you that research grant."

A response to Cobbs' initial findings in a 2004 issue of the journal Modern Pathology arrived from City of Hope researchers in Southern California that was particularly authoritative. The group studied 22 brain tumors and concluded that "none demonstrated evidence of CMV."

But Cobbs and his lab assistant had invented a more thorough technique to search for the virus, called antigen retrieval, which made the testing methodology 10 times more sensitive, with the result that the antibodies could more easily "see" the virus.

Cobbs said he offered to demonstrate the antigen retrieval technique to City of Hope researchers, but they declined to meet with him. They also did not respond to an interview request for this article.

**Extending patients' lives**

For the researchers who have confirmed Cobbs' methods, action is already under way. Mitchell, the Duke researcher, started a trial two years ago with 13 patients using a vaccine against the virus that stimulates the immune system to attack the infected tumor cells.

One of the desired results of such antiviral treatment is to thwart the return of cancerous tumors once they've been removed. Currently, according to the National Brain Tumor Society, about 95 percent of patients whose tumors were removed and who underwent chemotherapy and radiation treatment saw a tumor return in six to eight months.

Several of the patients in the Duke trial were tumor-free after two years, but Mitchell would not give specific data about his patients, which he is compiling for publication in a peer-reviewed journal. About half were just now seeing a return of the tumor, he said.

Doctors for Sen. Edward Kennedy, who was diagnosed with glioma in May and operated on at Duke, would not confirm whether he is participating in the anti-CMV drug trials at the university.

In San Francisco, some of Cobbs' patients who are taking Valcyte, an anti-CMV drug are experiencing results similar to those of Mitchell's patients.

Francis Gates, 81, who had a golf-ball-size tumor removed one year ago, only recently has seen signs of returning growth. Gates, who grew up in the rural Placer County town of Loomis, and worked as director of law libraries at University of Southern California and Columbia University, has undergone chemotherapy and radiation treatments in the past year in addition...
to taking Valcyte. Although he is unsure if his early life growing up on a farm matched the hygiene profile Cobbs had observed among glioma patients, he would not rule out the theory. He is also unsure if the antiviral drug is responsible for suppressing the tumor growth and extending his life, though he's thankful he's lived longer than doctors estimated.

"I feel lucky," Gates said. "But I've also felt that way my whole life. I just try to focus on my quality of life today, and see if I can't have some fun along the way."

Hopes for treatment, funding

During a recent two-hour surgery at California Pacific Medical Center on Castro Street, Cobbs removed a knot of infected brain tissue from an 83-year-old female patient and quickly dropped it into a vial held by Liliana Soroceanu, a neuroscience brain tumor researcher who then drove the tumor across town to their South of Market lab.

While Cobbs gathers research, removing roughly 100 tumors a year, it's still unknown how his CMV-related discovery will translate into treatment, especially for older patients, said Dr. Susan Chang, director of the Division of Neuro-Oncology at UCSF.

"There's still a number of questions to be answered as to what kind of treatments this discovery will lead to," Chang said, "and which patient populations might benefit most. Will this work best for older patients? Or only young patients? We're still not sure, and that's what we'll start discussing at the symposium."

The Boston gathering has been described as somewhat unusual by Cobbs' peers. Rarely is a surgeon able to gather so many top thinkers and researchers from multiple fields to discuss a theory that is not yet widely accepted by the medical establishment.

He hopes the event will also kick-start a critical mass of acceptance, so that funding will follow.

"We'll get the skeptics together, have a meeting, brainstorm and see if this is worth pursuing," Cobbs said.

"It's almost been taboo because no one wants to stick their neck out on this," he said referring to virologists and pathologists. "But I have nothing to lose. I'm just a surgeon."

Health: Business Wire founder Lorry Lokey donates $75 million to Stanford for stem cell research. B1

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http://sfgate.com/cgi-bin/article.cgi?f=/c/a/2008/10/06/MNJ8135E2I.1.DTL
Infections could contribute to adult brain tumours

24.01.2006

Infections could play a key role in triggering certain types of adult brain cancer, according to results from a new statistical analysis of the disease.

The international study, led by Dr Richard McNally at the University of Newcastle upon Tyne, was funded by Cancer Research UK, the Dutch Cancer Society, and the Christie Hospital Research Endowment Fund.

The British and Dutch team* analysed a database of adult brain tumours diagnosed in patients from the North Brabant province of the Netherlands between 1983 and 2001. They found clusters of cases of glioma tumours, which make up about half of all brain tumours, at different time intervals in different geographical locations.

This 'space-time clustering' of cases is a pattern typical of diseases caused by infections, adding weight to the theory that outbreaks of viruses are a potential contributory cause of brain tumours. Diseases caused by more constant environmental factors, such as pollution, produce clusters of cases in one place over a much longer time period.

However, the research team stresses it is too early to say exactly which infections could be the cause, and say that more research is needed to pinpoint what they are.

The findings, published in the European Journal of Cancer, may potentially be a step towards developing better preventative measures for cancer and may also result in better treatment.

Dr Richard McNally, of Newcastle University's School of Clinical Medical Sciences (Child Health), said: "Very little is known about the cause of brain tumours and we think our research brings us closer to understanding more about this disease.

"We only found clustering of cases in the East of the province we investigated, and we think it could be something to do with the way infections spread in less densely populated areas."

However, the researchers stress that people cannot 'catch cancer'. Infections are only likely to trigger cancer in a very small number of individuals who are already genetically susceptible to the disease.

The team looked at data from the Eindhoven Cancer Registry. They analysed results for the East and West of the 5000 square-kilometre province separately. They found an irregular pattern where many cases occurred at the same time in men and women over 15-years old in the East but not in the West. Around seven per cent more cases of brain tumours were observed to occur in 'clusters' than would be expected by chance.

This is the first study to carry out this sophisticated form of statistical analysis known as 'clustering' in brain tumours in adults.

The results of this work are consistent with earlier research led by Dr McNally into childhood cancer in North West England. This discovered patterns in the diagnosis of two types of cancer - leukaemia and brain tumours - in that they tended to occur together at similar times and geographical locations.

Dr McNally, who also works with Newcastle University’s School of Population and Health Sciences, added: "Future research should try to identify specific infections which could potentially be a trigger. If these are found, it could lead to future preventative measures."

Professor John Toy, Cancer Research UK's medical director, said: "Brain cancer is rare, accounting for less than two per cent of all new adult cancers diagnosed in the UK each year. These findings suggest a possible link
between infection and this type of the disease but by no means provide proof.”

Dr Richard McNally | Source: alphagalileo
Further information: www.newcastle.ac.uk
Space–time clustering patterns of gliomas in the Netherlands suggest an infectious aetiology

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Abstract

To test the hypothesis that infectious exposures may be involved in glioma aetiology, we have analysed space–time clustering and seasonal variation using population-based data from the South of the Netherlands between 1983 and 2001. Knox tests for space–time interactions between cases were applied, with spatial coordinates of the addresses at time of diagnosis, and with distance to the Nth nearest neighbour. Data were also analysed by a second order procedure based on K-functions. Tests for heterogeneity and Edwards’ test for sinusoidal variation were applied to examine seasonal variation of incidence. There was statistically significant space–time clustering in the Eastern, but not in the Western part of the region. Clustering was only present in adults, particularly in less densely populated areas. There was no evidence for seasonal variation. The results support a role for infectious exposures in glioma aetiology that may act preferentially in certain geographical areas.

Keywords: Aetiology; Glioma; Infection; Seasonal variation; Space–time clustering

1. Introduction

Gliomas are the most common primary brain tumours in children and adults. Thus far only ionising radiation has been established as an aetiological factor [1,2], and few genetic syndromes exist which predispose to glioma [3,4]. These factors however can only explain a small minority of cases, whilst the evidence for many other proposed risk factors is inconclusive [5]. A role for infection in the aetiology of glioma has been suggested. Certain viruses, including polyomaviruses, JC virus, BK virus and simian virus 40 (SV40) have been considered as possible aetiological agents but the findings have been inconsistent [6,7]. If infectious exposures are involved, the distribution of cases may exhibit space–time clustering. This would happen if an aetologically linked infectious exposure occurred in ‘mini–epidemics’ and would be detected when the lag time from exposure to diagnosis is short or relatively constant.

Space–time clustering is said to occur when excess numbers of cases are observed within small geographical locations at limited periods of time that cannot be
explained in terms of general excesses in those locations or at those times. The presence of seasonal variation would also provide indirect evidence for an aetiology involving infections that exhibited seasonal epidemiology. Examples of infections that display such epidemicity include the common cold, influenza and measles. Space–time clustering has been examined previously for childhood brain tumours using data from the Manchester Children’s Tumour Registry (MCTR). Statistically significant evidence for space–time clustering was found, particularly for astrocytoma and ependymoma, with an excess of patients born in Autumn or Winter [8]. However, in a study investigating childhood astrocytoma in Sweden, space–time clustering could not be shown [9]. To date, studies examining space–time clustering in adult glioma have not been published.

In the present study, we investigated space–time clustering and seasonal variation in adult and childhood glioma to assess the possibility of an infectious aetiology, using population-based data from cancer registries in the South of the Netherlands.

2. Patients and methods

The Eindhoven Cancer Registry within the framework of the Comprehensive Cancer Centre South and the Cancer Registry of Rotterdam registered all glioma patients in North Brabant. This province in the South of the Netherlands has 2.3 million inhabitants and covers an area of nearly 5000 km$^2$. The cancer registries in the Netherlands are characterised by high quality incidence data and near complete ascertainment [10,11]. Data were available for 1983–2001 for the Eastern part and 1989–2001 for the Western part of the province (the Eastern and Western parts are contiguous). To avoid any methodological bias, the Eastern and Western areas were analysed separately. All cases diagnosed with a central nervous system glioma were analysed.

For each case of glioma, geographical coordinates were allocated to the postcode of the address at the time of diagnosis. The geographical coordinates were obtained using the Dutch Triangular System (Rijksdriehoeksmeting: www.rdnap.nl), the most widely used geographical reference system in the Netherlands. This enabled spatial referencing of the Easting and Northing coordinates to within 0.1 km of the actual address. For $7\%$ of the cases in the total area of North Brabant and for all of those in a small region in the most Western part of the Western area, only partial postcodes (4 digits) were available, locating cases to the level of neighbourhoods and small municipalities. For these cases a random coordinate was used within the specified area. Sensitivity analyses were performed by repeating the analyses with another two different random coordinates. This created three data sets for analysis.

The following aetiological hypotheses were tested: (i) a primary factor influencing geographical or temporal heterogeneity of incidence of gliomas is related to exposure to an infectious or other similarly occurring environmental agent relatively close to disease onset; and (ii) geographical or temporal heterogeneity of incidence of gliomas is modulated by differences in patterns of exposure related to level of population density. Space–time interactions based on time and place of diagnosis were tested.

Knox space–time clustering tests were applied to the data with thresholds fixed, a priori, as: close in space, less than 5 km; and close in time, less than one year apart [12]. These limits are arbitrary, but have been used in a number of studies of space–time clustering of childhood cancers from North West England [8,13–16]. Furthermore, this problem is overcome by using the $K$-function method (see below). In the Knox test, a pair of cases is regarded as being in ‘close proximity’ if they are both diagnosed at addresses that are simultaneously close in space and at times that are close. The number of pairs of cases observed to be in close proximity was obtained ($O$) and the number of pairs of cases expected to be in close proximity was calculated ($E$). If $O$ exceeded $E$ there was space–time clustering and statistical tests were used to determine whether this excess was statistically significant. The magnitude of the excess (or deficit) was estimated by calculating $S=\left((O-E)/E\right) \times 100$. To adjust for the effect of different population densities, the tests were repeated replacing geographical distance thresholds by distance to the $N$th nearest neighbour, using all locations of all the cases in the data set. $N$ was chosen such that the mean distance was 5 km and was found to be $N=30$.

Two problems are apparent with the Knox test. First, boundary problems may be important since it can be impossible, or less probable, for some cases to be close in one dimension to other cases. The second problem concerns the arbitrariness of the thresholds chosen. A simplification of a second order procedure based on $K$-functions was used in the present analyses to overcome the problem of arbitrary boundaries [17]. This procedure involved a set of 225 Knox-type calculations where the boundaries changed over a pre-specified set of values (for close times, $t = 0.1, 0.2, \ldots, 1.5$ years and for close in space, $s = 0.5, 1, 1.5, \ldots, 7.5$ km). Statistical significance was assessed by simulation. Nearest neighbour (NN) approaches were also used (analogous to those described in relation to classical Knox tests).

Two age-groups were studied: 0–14, 15+ years. These age-groups were selected to attempt to differentiate between the potential effect of infectious exposures for children and older cases. For younger cases, genetic predisposition would be predicted to be an important component of aetiology in combination with the triggering infectious exposure, whilst for older cases the main
aetiological factor would be predicted to be the infectious exposure that precipitates the onset of the tumour.

To test the effect of the opportunity for exposure to infectious agents, via closer person to person contact, analyses were performed for two levels of population density. Addresses were classified as being located in a more densely populated area, or being located in a less densely populated area. For addresses at time of diagnosis the median distance for the 30th nearest neighbour was found. Diagnosis locations, whose 30th nearest neighbour was less than the median distance, were classified in the ‘more densely populated’ category. Diagnosis locations, whose 30th nearest neighbour was greater than the median distance, were classified in the ‘less densely populated’ category. Analysis was undertaken by considering pairs of cases including at least one case from the ‘more densely populated’ category and pairs of cases including at least one case from the ‘less densely populated’ category. The observed and expected numbers of pairs of cases were calculated where: (i) both cases came from a ‘more densely populated’ area; (ii) both cases came from a ‘less densely populated’ area; and (iii) one case came from a ‘more densely populated’ area and the other case came from a ‘less densely populated’ area. It should be noted that these analyses (especially the analyses of clustering pairs including at least one case from the ‘less densely populated’ category) are potentially subject to a strong diluting influence from edge effects since neither the ‘more densely populated’ areas nor the ‘less densely populated’ areas form a single spatially contiguous zone.

Of the three data sets, for all the analyses, the most conservative results in terms of \( P \)-value and for the Knox test, strength \( S \) within \( P \)-value are presented in tables. Statistical significance was indicated if \( P < 0.05 \), using at least 2 of the 4 methods (the geographical or NN version of the Knox test and the \( K \)-function method), and including a NN threshold version.

To examine seasonal variation the cases were examined for monthly variation in dates of birth and diagnosis using: (i) a \( \chi^2 \) test for heterogeneity, and (ii) Edwards’ test for sinusoidal variation [18]. The overall distribution of months of birth and diagnosis of all cancer patients registered by the Eindhoven Cancer Registry were used to correct the underlying variation in birth and diagnosis dates.

3. Results

For the province of North Brabant there were 1545 cases of glioma diagnosed between 1983 and 2001 (59.5% males, median age at diagnosis 52 years, range 0–92). There were 37 cases of pilocytic astrocytoma, 1064 cases of other astrocytoma, 131 cases of oligodendroglioma, 79 cases of ependymoma and 234 cases of other glioma including glioma not otherwise specified (NOS) and clinically diagnosed tumours. There were 124 cases of glioma in the most Western part of the Western area with only partial postcodes available.

There was statistically significant space–time clustering for cases from the Eastern, but not for cases from the Western part of the province \( (P < 0.05 \) using at least 2 methods and including a NN threshold version; Table 1). Statistically significant space–time clustering was found for cases of glioma aged over 15 years \( (P < 0.05 \) using at least 2 methods and including a NN threshold version), but not for children aged 0–14 years. Again this was apparent for cases from the East but not the West (Table 2). There was also no cross-clustering between the older (aged 15+ years) and younger cases (aged 0–14 years). When testing

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Table 1: Space–time clustering tests for glioma cases (all ages) in the South of the Netherlands and diagnosed during the period 1983–2001, analysed by area and time period.

<table>
<thead>
<tr>
<th>Area and time period (number of cases)</th>
<th>Knox test (observed space–time pairs, ( S ), expected space–time pairs, ( P )-value)</th>
<th>( K )-function analysis ( \times ) (( P )-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Geographical distance ( d )</td>
<td>NN threshold ( t )</td>
</tr>
<tr>
<td>East 1983–2001 (752 cases)</td>
<td>( O = 2550; E = 2482.9 )</td>
<td>( S = 2.7% ; P = 0.09 )</td>
</tr>
<tr>
<td>West 1989–2001 (793 cases)</td>
<td>( O = 2851; E = 2784.8 )</td>
<td>( S = 2.4% ; P = 0.11 )</td>
</tr>
</tbody>
</table>

\( a \) Cases are close in time if dates of diagnosis differ by less than 1 year.

\( b \) Strength \( S = \left| \frac{\text{Observed} - \text{Expected}}{\text{Expected}} \right| \times 100 \) counts of pairs which are close in time and space.

\( c \) 1-sided \( P \)-value derived from the Poisson distribution.

\( d \) When using geographical distance cases are close in space if their locations are <5 km apart.

\( e \) When using nearest neighbour (NN) thresholds cases are close in space if the locations of one (or both) is nearer than the other’s 30th NN in the total data set.

\( f \) Cases are close in time if dates differ by \( <t \), where \( t \) is in the range 1–18 months.

\( g \) \( P \)-value obtained by simulation (999 runs) with dates of diagnosis randomly re-allocated to the cases in the analysis.

\( h \) Cases are close in space if distances between their locations differ by \( <s \), where \( s \) is in the range 0.5–7.5 km.

\( i \) Cases are close in space if either is within the distance to the \( N \)th nearest neighbour of the other (in the total data set), where \( N \) is in the range 23–37.
for population density there was statistically significant space–time clustering involving cases from ‘less’ densely populated areas in the East but not the West (Table 3). Finally, there was no evidence of seasonal variation within both age groups using either the $\chi^2$ test for heterogeneity or Edwards’ test for sinusoidal variation (data not shown).

### 4. Discussion

To our knowledge, we are the first to apply formal statistical methods on population-based incidence data to study space–time clustering in adult glioma. Space–time clustering based on time and place of diagnosis was found. Clustering was only present in adults (aged

### Table 2

Space–time clustering tests for glioma cases in the South of the Netherlands and diagnosed during the period 1983–2001, analysed by age-group, area and time period

<table>
<thead>
<tr>
<th>Age-group, area and time period (number of cases)</th>
<th>Knox test (observed space–time pairs$, E$; expected space–time pairs, strength$b$, $P$-value$c$)</th>
<th>$K$-function analysis$^d$ ($P$-value$^e$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Geographical distance$^a$</td>
<td>NN threshold$^f$</td>
</tr>
<tr>
<td>East, 1983–2001 Age 0–14, (56)</td>
<td>$O = 8; E = 10.5$; $S = −23.5%$; $P = 0.72$</td>
<td>$O = 6; E = 8.6$</td>
</tr>
<tr>
<td></td>
<td>$O = 8; E = 10.5$; $S = −23.5%$; $P = 0.72$</td>
<td>$O = 6; E = 8.6$</td>
</tr>
<tr>
<td>Age 15+, (696)</td>
<td>$O = 2239; E = 2161.2$; $S = 3.6%$; $P = 0.05$</td>
<td>$O = 1440; E = 1335.6$</td>
</tr>
<tr>
<td>West, 1989–2001 Age 0–14, (45)</td>
<td>$O = 9; E = 10$; $S = −9.6%$; $P = 0.54$</td>
<td>$O = 4; E = 7.8$</td>
</tr>
<tr>
<td></td>
<td>$O = 9; E = 10$; $S = −9.6%$; $P = 0.54$</td>
<td>$O = 4; E = 7.8$</td>
</tr>
<tr>
<td>Age 15+, (748)</td>
<td>$O = 2543; E = 2493.6$; $S = 2.0%$; $P = 0.16$</td>
<td>$O = 2162; E = 2120.3$</td>
</tr>
<tr>
<td></td>
<td>$O = 2543; E = 2493.6$; $S = 2.0%$; $P = 0.16$</td>
<td>$O = 2162; E = 2120.3$</td>
</tr>
</tbody>
</table>

$^a$ Cases are close in time if dates of diagnosis differ by less than 1 year.

$b$ Strength ($S$) = $\{(Observed − Expected)/Expected\} \times 100$ counts of pairs which are close in time and space.

$c$ 1-sided $P$-value derived from the Poisson distribution.

$d$ When using geographical distance cases are close in space if their locations are <5 km apart.

$e$ When using nearest neighbour (NN) thresholds cases are close in space if either is within the distance to the $N$th nearest neighbour of the other (in the total data set), where $N$ is in the range 23–37.

$^f$ Case is close in time if dates differ by $t$, where $t$ is in the range 1–18 months.

$^g$ $P$-value obtained by simulation (999 runs) with dates of diagnosis randomly re-allocated to the cases in the analysis.

$h$ Cases are close in space if either is within the distance to the $N$th nearest neighbour of the other (in the total data set), where $N$ is in the range 23–37.

### Table 3

Space–time clustering tests for glioma cases in the South of the Netherlands and diagnosed during the period 1983–2001, analysed by population density, area and time period

<table>
<thead>
<tr>
<th>Population density, area and time period</th>
<th>Knox test (observed space–time pairs$, E$; expected space–time pairs, strength$b$, $P$-value$c$)</th>
<th>$K$-function analysis$^d$ ($P$-value$^e$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Geographical distance$^a$</td>
<td>NN threshold$^f$</td>
</tr>
<tr>
<td>East, 1983–2001 MDP$^i$</td>
<td>$O = 2221; E = 2172.3$; $S = 2.2%$; $P = 0.15$</td>
<td>$O = 1039; E = 1002.9$</td>
</tr>
<tr>
<td></td>
<td>$O = 2221; E = 2172.3$; $S = 2.2%$; $P = 0.15$</td>
<td>$O = 1039; E = 1002.9$</td>
</tr>
<tr>
<td>LDP$^j$</td>
<td>$O = 463; E = 444.3$; $S = 4.2%$; $P = 0.19$</td>
<td>$O = 823; E = 751.1$</td>
</tr>
<tr>
<td></td>
<td>$O = 463; E = 444.3$; $S = 4.2%$; $P = 0.19$</td>
<td>$O = 823; E = 751.1$</td>
</tr>
<tr>
<td>West, 1989–2001 MDP$^i$</td>
<td>$O = 2385; E = 2353.8$; $S = 2.1%$; $P = 0.16$</td>
<td>$O = 1486; E = 1459.1$</td>
</tr>
<tr>
<td></td>
<td>$O = 2385; E = 2353.8$; $S = 2.1%$; $P = 0.16$</td>
<td>$O = 1486; E = 1459.1$</td>
</tr>
<tr>
<td>LDP$^j$</td>
<td>$O = 791; E = 786.0$; $S = 0.6%$; $P = 0.43$</td>
<td>$O = 1370; E = 1362.9$</td>
</tr>
<tr>
<td></td>
<td>$O = 791; E = 786.0$; $S = 0.6%$; $P = 0.43$</td>
<td>$O = 1370; E = 1362.9$</td>
</tr>
</tbody>
</table>

$^a$ Cases are close in time if dates of diagnosis differ by less than 1 year.

$b$ Strength ($S$) = $\{(Observed − Expected)/Expected\} \times 100$ counts of pairs which are close in time and space.

$c$ 1-sided $P$-value derived from the Poisson distribution.

$d$ When using geographical distance cases are close in space if their locations are <5 km apart.

$e$ When using nearest neighbour (NN) thresholds cases are close in space if either is within the distance to the $N$th nearest neighbour of the other (in the total data set), where $N$ is in the range 23–37.

$f$ Case is close in time if dates differ by $t$, where $t$ is in the range 1–18 months.

$g$ $P$-value obtained by simulation (999 runs) with dates of diagnosis randomly re-allocated to the cases in the analysis.

$h$ Cases are close in space if distances between their locations differ by <5 km.

$i$ Cases are close in space if either is within the distance to the $N$th nearest neighbour of the other (in the total data set), where $N$ is in the range 23–37.

$j$ $\geq 1$ case from a more densely populated (MDP) area.

$k$ $\geq 1$ case from a less densely populated (LDP) area.
The current data set. Population growth are not thought to be important in whether real or artifactual. Additionally, variations in month that are not available. However, it must be stressed that the current analyses provide a description of the space–time clustering patterns in the data, whether real or artifactual. Additionally, variations in population growth are not thought to be important in the current data set.

The pattern of space–time clustering found in this study is consistent with an exposure occurring at a relatively short time period before onset of the disease. It is likely that this exposure is more important among those aged over 15 years. The nature of space–time interaction implies an exposure emerging at many points in both place and time. Therefore, more sustained exposures which are geographically fixed and present for long periods of time (e.g., power lines, environmental pollution or industry) can be excluded. The pattern is however more consistent with an infectious agent. Since there was only space–time clustering in the Eastern part of the province, this agent is likely to act in limited geographical areas, without spreading to other regions. This would imply that this agent does not have the capability for rapid spreading, or that it is linked to, e.g., industries or environments that are more common in the East. The more marked clustering in less densely populated areas might indicate that the aetiological agent is more prevalent in these environments. We however do not know of any common industry or environment that is typical for the Eastern part of the province of North Brabant.

Evidence for the involvement of infections in the aetiology of glioma comes primarily from studies in experimental animals and from the isolation of several viruses from human tumour material. The importance of these findings to glioma aetiology is uncertain. Few epidemiological studies addressing the role of infections have been published, which may also indicate unpublished negative results. For adult glioma, antibody titres to Toxoplasma gondii were linked to astrocytoma [20], although an association could not be confirmed by others [21]. For childhood glioma, four epidemiological studies suggested an infectious component to aetiology [22–25], whilst another case–control study found no such relations [26].

No studies concerning space–time clustering in adults have been published thus far. Therefore comparisons can only be made for childhood brain tumours. In the present study, no clustering was detected for the youngest age category. Also, no space–time clustering was found in childhood astrocytoma in Sweden [9]. Space–time clustering was however reported for childhood brain tumours using population-based data from the Manchester Children’s Tumour Registry (MCTR) [8].

Strong evidence for space–time clustering was found for astrocytoma, ependymoma and all glioma combined. The present study contained far fewer cases of childhood glioma than the MCTR study, whilst the Swedish study used a different methodology. It is possible that the lack of space–time clustering in the present study is due to insufficient power to detect such an effect.

We found no evidence for seasonal variation in glioma incidence. In earlier studies however, seasonal variation was observed for childhood astrocytoma and ependymoma [8], for all childhood brain tumours [27],...
and for adult glioma [28]. All studies reported excesses in incidence for late autumn and winter births. The first two studies concerned childhood glioma and brain tumours only, probably explaining most of the discrepancies with the present study in which there was insufficient power owing to a lack of childhood cases. The third study investigating adult glioma used a different methodology. Furthermore, we used a robust method of adjusting variations in birth and diagnosis date with the overall distribution of months of birth and diagnosis for all cancer patients registered by the cancer registry.

In summary, space–time clustering was found for cases of glioma from the Eastern part of the province, but only for adults aged >15 years. The results are consistent with an infectious agent, mainly acting in limited, less densely populated geographical areas without spreading to other regions. It is difficult to draw any firm conclusions concerning the childhood cases (aged 0–14 years), due to small numbers.

It is not clear whether there are one or more candidate infections or whether infectious agents in general act as a tumour promoter. Further research should include both epidemiological and laboratory investigations. An ecological investigation could relate incidence rates to levels of deprivation and studies of spatial clustering could determine if there are small areas with sustained high incidence. Laboratory studies might examine differences in the occurrence of specific putative agents between ‘clustering’ and ‘non-clustering’ cases.

Conflict of interest statement

The authors have no conflicts of interest to disclose.

Acknowledgements

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Virus Mimics Human Protein To Hijack Cell Division Machinery

*ScienceDaily* (May 10, 2008) — Viruses are masters of deception, duping their host’s cells into helping them grow and spread. A new study has found that human cytomegalovirus (HCMV) can mimic a common regulatory protein to hijack normal cell growth machinery, disrupting a cell’s primary anti-cancer mechanism.

Writing in the May 9 issue of *Science*, researchers from the University of Wisconsin-Madison and Harvard Medical School report that a viral protein, called UL97, masquerades as a normal regulatory enzyme to modify a tumor-suppressing protein in human cells. Unlike the normal enzyme, which can be switched on and off by the cell as needed, the viral stand-in lacks an off switch and evades cellular control. The findings represent a previously unknown way that viruses can cause uncontrolled cell growth and division.

Cells normally have tight regulatory mechanisms in place to limit multiplication to appropriate situations, such as replacing worn-out cells or repairing damage. Uncontrolled cell proliferation can lead to cancer and other disorders.

One of the most important cellular control mechanisms works through a protein called the retinoblastoma tumor suppressor protein, which slows cell growth.

“The retinoblastoma pathway is like the brakes on a car. It prevents tumor cells from growing out of control,” says Robert Kalejta, an assistant professor in the UW-Madison Institute for Molecular Virology and McArdle Laboratory for Cancer Research, who led the new study. “This pathway is mutated in essentially all human cancers.”

Disrupting this pathway is also advantageous for viruses. Unable to reproduce on their own, viruses rely on co-opting their host’s cellular machinery, like an occupying army taking over a local factory. They are especially good at overriding or bypassing built-in control mechanisms, Kalejta says.

“Viruses are well known to encode proteins that have similar abilities,” Kalejta says. “But this is a completely new mechanism for hijacking cellular systems.”

The findings represent a previously unknown way that viruses can cause uncontrolled cell growth and division. The researchers say that the results could help scientists develop new treatments for cancer and other diseases.

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In essence, UL97 disables the brakes and hits the gas. Once a host cell is primed toward growth, HCMV takes over and steals the cell's machinery to reproduce itself.

The virus's bloodhound-like ability to seek out and target the most essential pieces of a cell's machinery makes it a valuable research tool, Kalejta says. "Viruses are smarter than we are. They know a lot more about cells than we do, because their life depends on it - they're obligate intracellular parasites," he says. "If they attack a part of the cell - a process or a protein - you know it's important for the cell. If the virus pays attention to it, you should too."

Kalejta next hopes to use UL97 to find other proteins that may be important for cell growth. He also sees potential clinical applications down the road. HCMV infection is very common and, though it remains asymptomatic in most people, it has been implicated in some cancers and can cause trouble in people with compromised or suppressed immune systems, such as AIDS patients and transplant recipients. In addition, UL97-like proteins are also found in the other seven human herpes viruses, some of which are directly linked to cancers.

The advantages of the research are two-fold, Kalejta says. "We're studying a virus that causes human disease and might eventually find a way to treat that infection and help patients. At the same time, we're learning about how the cell works, which has implications for patients that don't have infections," he says. "You get two for the price of one."

Other authors on the paper include Adam Hume, Jonathan Finkel, and Michael Culbertson from UW-Madison and Jeremy Kamil and Donald Coen from Harvard Medical School. The work was funded by grants from the National Institutes of Health, the Wisconsin Partnership for a Healthy Future, the Burroughs Wellcome Fund, and the American Heart Association.

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Phosphorylation of Retinoblastoma Protein by Viral Protein with Cyclin-Dependent Kinase Function

Adam J. Hume,† Jonathan S. Finkel,‡ Jeremy P. Kamil,§ Donald M. Coen,∥ Michael R. Culbertson,‡ Robert F. Kalejta†*

As obligate intracellular parasites, viruses expertly modify cellular processes to facilitate their replication and spread, often by encoding genes that mimic the functions of cellular proteins while lacking regulatory features that modify their activity. We show that the human cytomegalovirus UL97 protein has activities similar to cellular cyclin–cyclin-dependent kinase (CDK) complexes. UL97 phosphorylated and inactivated the retinoblastoma tumor suppressor, stimulated cell cycle progression in mammalian cells, and rescued proliferation of Saccharomyces cerevisiae lacking CDK activity. UL97 is not inhibited by the CDK inhibitor p21 and lacks amino acid residues conserved in the CDKs that permit the attenuation of kinase activity. Thus, UL97 represents a functional ortholog of cellular CDKs that is immune from normal CDK control mechanisms.

Cyclin–cyclin-dependent kinase (CDK) complexes are found in all eukaryotes and control cell cycle progression and other processes (1). In higher eukaryotes, a major target of the CDKs is the retinoblastoma (Rb) tumor-suppressor protein that controls progression through G1 phase of the cell cycle. The pathway controlled by Rb may be aberrant in most human cancers (2). Unphosphorylated Rb binds to E2F transcription factors, thus inhibiting the expression of genes required for DNA replication and arresting cell cycle progression in G0 or G1 phase. During normal cell cycle progression, Rb is functionally inactivated by multiple phosphorylations mediated sequentially by a series of CDK complexes (3). Phosphorylation of Rb disrupts complexes with E2Fs, allowing for cell cycle progression into S phase. To create an advantageous cellular environment for viral replication, viruses can inactivate Rb through direct binding of viral proteins to Rb and the consequent disruption of Rb–E2F complexes, by causing Rb degradation, or through constitutive activation of cellular CDKs by virally encoded cyclin proteins (4, 5). Here, we describe a virally encoded protein kinase that directly phosphorylates Rb, and we show that this kinase can substitute for CDKs during cell cycle regulation.

Upon infection of quiescent cells with human cytomegalovirus (HCMV), unphosphorylated Rb is first degraded by pp71 (6) and then phosphorylated (7, 8) (Fig. 1A and fig. S1). Phosphorylated Rb migrates more slowly during SDS–polyacrylamide gel electrophoresis than does the unphosphorylated form of the protein, and it can be detected in lysates from HCMV-infected cells within 4 hours after infection (Fig. 1A). Three small-molecule inhibitors of CDK activity (roscovitine, olomoucine, and flavopiridol) inhibited Rb phosphorylation induced by serum stimulation, but not phosphorylation induced by HCMV infection (Fig. 1B). Additional experiments with a panel of 20 kinase inhibitors (table S1) revealed that only 2, G06976 and NGIC-I, inhibited Rb phosphorylation during HCMV infection (Fig. 1C). These drugs inhibit both cellular protein kinase C (PKC) and the HCMV protein kinase UL97 (9). However, G07478, an inhibitor of PKC that does not inhibit UL97, did not reduce Rb phosphorylation in HCMV-infected cells (Fig. 1C). Because an HCMV mutant lacking the UL97 gene (10) did not induce Rb phosphorylation (Fig. 1, D and E) and because of the presence of three potential Rb-binding motifs in UL97 (fig. S2), we suspected that UL97 was required for Rb phosphorylation during HCMV infection. Phosphorylation of Rb on Ser780, Ser807, Ser811, and Thr821 inactivates the cell cycle–inhibitory and tumor-suppressor functions of Rb by disrupting Rb–E2F complexes (3). All of these residues are phosphorylated in HCMV-infected cells (Fig. 2A). Residues not known to modify Rb function upon phosphorylation, such as Ser259 and Thr252, were not phosphorylated in HCMV-infected cells but were phosphorylated in serum-stimulated cells (Fig. 2A). A recombinant HCMV in which the wild-type (WT) UL97 gene was replaced with an allele encoding a UL97 protein substituted at the active site Lys (Lys355 → Gin355; K355Q) failed to induce phosphorylation of Rb, but a WT revertant virus derived from the K355Q mutant did induce the phosphorylation of Rb (Fig. 2B). The UL97-K355Q mutant virus exhibited a growth defect similar to that of the UL97-null virus, and the growth defect was rescued in the revertant virus (fig. S3). The CDK inhibitor flavopiridol again failed to prevent HCMV-induced phosphorylation of Rb in HCMV-infected cells, but two drugs that inhibit UL97 kinase activity (G06976 and maribavir) did inhibit such phosphorylation (fig. S4). Thus, in HCMV-infected cells, kinase activity of UL97 is necessary for Rb phosphorylation on residues that inactivate its function. Rb degradation and phosphorylation in HCMV-infected cells are independent events (fig. S1).

We also tested whether UL97 alone is sufficient to induce Rb phosphorylation. Transfection of expression plasmids for epitope-tagged wild type [but not a catalytically inactive (Lys355 → Met355; K355M) mutant (11)] induced the phosphorylation of cotransfected Rb on inactivating residues (Fig. 3A) in Saos-2 cells that are intrinsically unable to phosphorylate Rb. Drugs that inhibit UL97 partially suppressed Rb phosphorylation when added to UL97-expressing...
Saos-2 cells, but those that inhibit the CDKs or only PKC did not (Fig. 3B). The K355M mutant protein was readily detected by protein immunoblotting, but WT UL97 was consistently expressed in lower amounts. Inhibition of kinase activity of WT UL97 with drugs also seemed to allow accumulation of more protein (Fig. 3B), perhaps indicating that active UL97 is toxic to mammalian cells. Nevertheless, UL97 expression was sufficient to induce Rb phosphorylation. Purified UL97 phosphorylated an Rb fragment in vitro, as assayed by the incorporation of $^{32}$P (Fig. 3C), or with an antibody that specifically recognized Rb phosphorylated on Ser$^{807}$ and Ser$^{811}$ (Fig. 3D). Autophosphorylation was also detected (fig. S5). UL97 showed the same spectrum of sensitivity to kinase inhibitors in vitro as was observed for Rb phosphorylation in HCMV-infected cells (Fig. 3, C and D), and purified catalytically inactive (K355Q) UL97 failed to phosphorylate Rb in vitro (Fig. 3, C and D). These results make it unlikely that a copurifying insect kinase phosphorylated Rb in this assay.

UL97 directly phosphorylates Rb on inactivating residues, an activity shared by cellular CDKs. Therefore, we tested whether UL97 represents a functional CDK ortholog by testing if

**Fig. 1.** Requirement of viral UL97 protein kinase, but not cellular CDKs, for Rb phosphorylation in HCMV-infected cells. (A) Subconfluent human fibroblasts deprived of serum (ssHFs) were mock-infected (M), infected with HCMV (V) at a multiplicity of infection (MOI) of 3, or stimulated with serum (S). Lysates prepared at the indicated times (in hours) were analyzed by Western blots. The Rb antibody (4H1) detects all forms of the protein. Virion-delivered pp71 (71) and newly synthesized IE1 are viral proteins, and tubulin (tub) is a loading control. (B) Cells treated as in (A) were also incubated with the CDK inhibitors roscovitine (R), olomoucine (O), or flavopiridol (F). Proteins in lysates harvested at 6 hours from virus-infected cells or 24 hours from uninfected cells were analyzed by Western blots. Dashes indicate no drug treatment. (C) ssHFs treated as in (A) were also incubated with the PKC inhibitors Gö6976 (1), Gö7874 (2), or NGIC-I (3), and proteins in lysates collected after 6 hours were analyzed by Western blots. Expression of the viral UL97 protein is confirmed with a UL97 specific antibody. (D) ssHFs were mock infected or infected with WT HCMV or a UL97-null virus (Δ97) at an MOI of 1. Proteins from lysates collected at the indicated hour were analyzed by Western blots. (E) ssHFs were treated as in (D), and proteins from lysates harvested on the indicated day after infection were analyzed by Western blots. Viral late gene expression was confirmed by detection of the viral pp28 protein (28).

**Fig. 2.** Phosphorylation of Rb on residues that inactivate its tumor-suppressor function in HCMV-infected cells. (A) ssHFs were mock infected (M), infected with HCMV (V) at an MOI of 3, or stimulated with serum (S). Proteins from lysates prepared at 24 hours were analyzed by Western blots with control antibodies (IE1 and tub) and antibodies specific for all forms of Rb (Rb), pp71 (pp71), and newly synthesized IE1 (IE1). (B) ssHFs were mock-infected, serum-stimulated, infected with a recombinant HCMV expressing a substituted form of UL97 (K355Q) that lacks kinase activity (K) or a WT revertant of the K355Q virus (R). Proteins from lysates harvested at 24 hours were analyzed by Western blots.

**Fig. 3.** Phosphorylation of Rb in vivo and in vitro by UL97. (A) Proteins from lysates of Saos-2 cells transfected with an Rb expression plasmid and either an empty vector (EV) or expression plasmids for V5-epitope–tagged WT (97) or catalytically inactive (KM) UL97 were analyzed by Western blots with the indicated antibodies. (B) Thirty hours after transfection as above, cells were left untreated (−) or treated with Gö6976 (G6), Gö7874 (G7), NGIC-I (NG), flavopiridol (F), maribavir (Mb), or roscovitine (R) for 18 hours before harvesting lysates and analysis of proteins by Western blots. (C) Purified GST-UL97 kinase was incubated in vitro with a His-tagged Rb fragment in a kinase reaction supplemented either with dimethyl sulfoxide (DMSO) (D) or the indicated drugs. Purified catalytically inactive GST-UL97-K355Q was also analyzed (K/Q). Transfer of radiolabeled phosphate ($^{32}$P) to Rb was detected by phosphorimaging, and total Rb was detected by Coomassie staining (Cm). (D) Samples from an in vitro kinase assay containing no added kinase (−), WT GST-UL97 (97), WT GST-UL97 plus maribavir (97M), or the catalytically inactive mutant UL97 (K/Q) were analyzed by Western blots for total Rb or Rb phosphorylated on Ser$^{807}$ and Ser$^{811}$ (807).
UL97 could rescue the cell cycle defect of yeast lacking CDK activity. A yeast mutant (12) with a temperature-sensitive allele of the single CDK gene (cdc28-13) arrests in G1 as unbudded cells at the restrictive temperature (Fig. 4A and fig. S6). Expression of human Cdk1 or WT UL97 allowed the cells to remain cycling at the restrictive temperature (Fig. 4A). Catalytically inactive UL97, or the viral pp71 protein (6), failed to rescue the cell cycle defect. UL97 also stimulated the cell cycle of mammalian cells (fig. S7). The functional properties presented here lead us to conclude that UL97 is a viral CDK functional ortholog.

We expanded an alignment (13) of the kinase subdomains of UL97 with four cellular Ser/Thr kinases (including Cdk2) to include the intervening sequences (fig. S8). Our analysis suggested that UL97 is not subject to the regulatory mechanisms that control CDK activity (14), and we confirmed that prediction. UL97 lacks most of the residues of Cdk2 that make contacts with cyclin A, including the conserved PSTAIRE (15) helix required for cyclin binding (16), indicating that UL97 is unlikely to bind to cyclins. Because cyclins did not copurify with UL97 during tandem affinity purification (17) and because of the high activity of UL97 on Rb in vitro (Fig. 3, C and D) in the absence of a cyclin, we conclude that UL97 does not require cyclin binding for activity. The affinity of CDKs for cyclins is enhanced by CDK activating kinase (CAK)—mediated phosphorylation (18) of CDKs on Thr(160). Neither the Thr nor any of the three Arg residues of CDKs that coordinate the phosphate (16) are conserved in UL97 (fig. S8). Because flavopiridol and roscovitine, both CAK inhibitors (18), do not inhibit the ability of UL97 to phosphorylate Rb in vivo (Figs. 1B and 3B and fig. S4), we conclude that CAK-mediated phosphorylation does not activate UL97. CDK activity is attenuated by phosphorylation on Tyr(15) during G2 phase of the cell cycle and in response to radiation (19). UL97 has a Phe substitution (fig. S8) and thus cannot be phosphorylated at this site. UL97 (fig. S8) lacks most of the conserved CDK residues (1, 14) that interact with members of both classes of CDK inhibitors (CKIs), p21, a potent CKI (20), did not efficiently inhibit UL97-mediated phosphorylation of Rb in vivo (Fig. 4B) or in vitro (Fig. 4C).

UL97 is a viral CDK ortholog that is immune from normal cellular control mechanisms that attenuate CDK activity and represents a previously unknown mechanism through which viruses regulate the cell cycle. The remote sequence similarity between CDKs and conserved herpesvirus protein kinases (CHPKs) such as UL97 led others to speculate that CHPKs may mimic CDK function (13). We provide direct experimental evidence that UL97 is functionally orthologous to cellular CDKs. UL97 is a target for anti-HCMV therapies because it phosphorylates (and thus activates) the antiviral drug ganciclovir (21) and because it is inhibited by maribavir (22). UL97 may also be useful as a tool to study the inactivation of the Rb pathway by phosphorylation, to identify other critical substrates of CDKs, and to probe the evolutionary relationships between viral and host cell kinases.

References and Notes


8. Materials and methods are available as supporting material on Science Online.


15. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Glu; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.


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Supporting Online Material

www.sciencemag.org/cgi/content/full/320/5877/797/DC1

Materials and Methods

Figs. S1 to S8

Table S1

References

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CMV infection in cancer

THE SIGNIFICANCE OF CMV INFECTION IN CANCER PATIENTS

Recent reports reveal the frequent presence of the genome and proteins of CMV in certain malignant tumors, such as colon cancer, malignant glioma, EBV-negative Hodgkin’s lymphoma, cervix cancer, prostate cancer and breast cancer. The frequency of CMV proteins in these cancer forms is remarkably high as >90% of tumor tissues are CMV positive. The expression is however low, as only optimized immunohistochemistry protocols can be used to identify viral proteins in tumors. In transplant patients, Lymphoproliferative disease (PTLD) is the most common malignancy and occurs in 1–20% of solid organ transplant patients. PTLD has been linked to high immunosuppression and reactivation of Epstein-Barr virus. However, the incidence of PTLD is also increased 7–10-fold in patients with CMV disease, and PTLD is associated with CMV reactivation. Prophylaxis with ganciclovir, which targets both viruses, reduces the risk of PTLD in organ transplant recipients.

We have confirmed the presence of an active CMV infection in 99% of malignant glioblastoma tumors (ms in revision), and also recently found an active CMV infection in approximately 90% of medulloblastomas (MB) and neuroblastoma (NB) tumors (2 manuscripts). In addition, as Cobbs group, we found the virus in >90% of tumor cells in breast cancer, prostate cancer, colon cancer and ovarian cancer. Importantly, the virus infection remains latent in non-cancer tissue specimens obtained from the same patient, and in healthy control individuals. This new and surprising information suggests that a persistently active and previously undiscovered CMV infection also appear to be present in many tumors. Whether CMV is causative or simply represents an epiphenomenon of malignant tumors urgently requires further elucidation.

CMV IS DETECTED IN GlioBLASTOMAS AND IS OF HIGH PROGNOSTIC VALUE FOR PATIENT SURVIVAL

Recently, we discovered that glioblastoma patients with less than 25% CMV infected tumor cells at diagnosis survived over 2 years (27.75 months) longer than patients with 25-90% CMV infected tumor cells (n=89, p<0.0014). At two years, approximately 60% of the patients with low-grade CMV infection in the tumor were alive, as compared with 20% of patients with high grade infection. Time to progression was 22 versus 9 months (p=0.0018). These observations strongly suggest that CMV has a pathogenetic role rather than being an epiphenomenon in malignant glioblastoma.

IS CMV AN ONCOCGENIC OR AN ONCOMODULATORY VIRUS?

In the 1970th, Fred Rapp’s group reported a repeated presence of CMV in prostate cancer, and isolated a virus strain from tumors that was oncogenic in animal models. In several later studies, CMV failed to transform normal human cells, so this virus was not considered to be oncogenic. Instead, the term oncomodulation has been proposed to describe the indirect influence of CMV on tumor development. Oncomodulation is defined as the ability to promote, in the appropriate genetic environment supplied by tumor cells, an oncogenic process characterized by disruptions in intracellular signaling pathways, transcription factors and tumor suppressor proteins. CMV can block cellular differentiation, interfere with oncogene expression, induce chromosomal instability specific chromosomal breaks, inhibit DNA repair mechanisms, control important epigenetic functions and cellular proliferation, inhibit apoptosis, induce angiogenesis and cellular migration that all provide oncomodulatory mechanisms. More recent data by M. Smits group suggest that the virus in fact also may be oncogenic; expression of the CMV protein US28 by itself leads to tumor development in a murine model through induced COX-2 expression and VEGF production. In addition, we recently found that the CMV protein IE72 induces high telomerase activity through binding and recruitment of SP-1 to the hTERT promoter. We also demonstrated hTERT expression in CMV infected cells in glioblastoma tumors (JNCI 2009). Induction of
telomerase activity is a key event in cancer development and a common phenomenon of oncogenic

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CAN CMV GENE PRODUCTS CONFER RESISTANCE TO CHEMOTHERAPY?

Apoptosis, or programmed cell death, is the final step in the mechanisms for killing mediated by NK cells and CTLs. Attenuated sensitivity of tumor cells to drug-induced death is one of the major reasons for the failure of anti-cancer therapy. At least six different CMV proteins (i.e., IE1, IE2, UL36 and UL37, UL38 and vMIA) inhibit apoptosis and could thereby enhance the survival of CMV-infected tumor cells, as well as their susceptibility to chemotherapy. In support of this hypothesis, UL36 expression in neuroblastoma cells confer resistance against chemotherapy in vitro.

CMV AVOIDS IMMUNE RECOGNITION

CMV has developed sophisticated mechanisms designed to avoid recognition by the immune system. For example, CMV inhibits the expression of HLA class I and class II molecules and antigen presentation, it controls T cell activation, inhibits NK cell activation and protects cells from cytolytic peptides that are released from activated T and NK cells. These are examples of strategies that make infected cells invisible to the immune system, and may explain why CMV infected tumors are not controlled by the immune system or by immunotherapies developed against them, as infected tumor cells will most likely be invisible to the immune system. Our group has discovered several CMV immune evasion strategies that may aid cancer avoid detection and elimination by the hosts immune system.

WILL ANTI-VIRAL DRUGS GIVE NEW HOPE FOR MALIGNANT GLIOBLASTOMA PATIENTS?

We have initiated treatment of 12 patients with recurrent glioblastoma with anti-viral drugs against CMV. Many of these patients have demonstrated unexpected remarkable clinical improvements. We have therefore recruited 42 patients into the world’s first clinical study (VIGAS; double blinded/proof of concept multi-center study, investigational grant supported by Roche) aiming at evaluating whether anti-viral drugs against CMV given as an add-on therapy to patients with malignant glioblastoma improves the prognosis for these patients. Current preliminary follow up data of these patients demonstrate a potential benefit of anti-CMV treatment in glioblastoma patients.

CMV INFECTION IN MALIGNANT PEDIATRIC TUMORS OF THE NERVOUS SYSTEM

Is has been demonstrated previously that neuroblastomas (NB) and medulloblastomas (MB) tumors express 5-LO and COX-2 and that 5-LO and COX-2 inhibitors can reduce tumor growth in animal models. Since CMV induces both COX-2 and 5-LO, and in a collaboration with professor Per Kogner we interestingly found that only the CMV infected tumor cells express COX-2 and 5-LO in NB and MB tumors. We are currently focusing on understanding whether CMV controls COX-2 and 5-LO expression in these tumors. Both COX-2 and 5-LO inhibitors decreased viral protein expression in vitro in persistently infected cells. In xenograft models of NB and MB, we found that
COX-2 inhibitors reduce tumor growth by 30%, the anti-CMV drug Valcyte by 35-40% and combination therapy results in 70-75% inhibition of tumor growth (MB, Figure 4) without chemotherapy. Thus, by interfering with virus replication in the tumors, the growth of the tumors is severely impaired and gives hope for new targeted therapies for patients with brain tumors.

Our project focuses on understanding the biological role of CMV in cancer. In animal models, we test different treatment combinations with future aim to develop new treatments for CMV infected cancer forms. Already, we are performing the world's first clinical trials on antiviral treatment in malignant glioblastoma patients.


Center for Molecular Medicine - for research on the common diseases

The Center for Molecular Medicine (CMM) at Karolinska Institutet gathers more than 400 staff members with clinical experience from Karolinska University Hospital and with research competence from KI for research on the common diseases. The ultimate aim of our work is to provide knowledge that leads to the development of new and improved prevention and therapy for common diseases such as rheumatic diseases, MS, psoriasis, cardiovascular diseases, several genetic diseases, and certain neuropsychiatric diseases such as depression or alcohol addiction.

The philosophy of CMM is to foster innovative research within the core areas of the center both from using unique features of our clinical connection (extensive clinical material and clinical competence) and from being part of KI with its international reputation and excellent basic research. A special feature of CMM is its international flavour, where more than 30 nationalities are now represented.

CMM is presently comprised of 28 research groups which comprise an interactive network of scientists focusing on molecular mechanisms behind inflammatory, cardiovascular, genetic and neuropsychiatric diseases. The center is formally an independent foundation which provides researchers from KI and Karolinska University Hospital with research laboratories and infrastructure for their research.

The CMM laboratory building is located at the interface between the Karolinska University Hospital, Solna, and Karolinska Institutet campus, with immediate contact with the clinical work, and with close contacts with the basic science departments at the campus.

Stockholm
Sweden
Recommendations from the
IHMF Management Strategies Workshop and
3rd Annual Meeting

Editors: Dr E Sandström
Professor RJ Whitley

THE INCREASING IMPORTANCE OF CYTOMEGALOVIRUS, EPSTEIN-BARR VIRUS AND THE HUMAN HERPESVIRUSES TYPES 6, 7 AND 8

Jointly sponsored by the University of Alabama School of Medicine,
University of Alabama at Birmingham, USA and
the International Herpes Management Forum (IHMF)

management strategies in herpes
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The above were participants in the Management Strategies Workshop.
The contribution of the participants at the 3rd Annual Meeting of the IHMF is also acknowledged.

management strategies in herpes
The International Herpes Management Forum (IHMF) was established to improve the awareness, understanding, counselling and management of infections caused by herpesviruses. Steered by an IHMF Board of Professor Richard Whitley, Dr Martin Wood, Dr Larry Corey, Professor Paul Griffiths, Dr Susanne Kroon, Dr Antonio Volpi and Dr Koichi Yaminishi, the IHMF involves international Opinion Leaders in all aspects of medical management of herpesvirus infections including herpes simplex virus (HSV), varicella zoster virus (VZV) and cytomegalovirus (CMV) infections.

Two Management Strategies Workshops were held in 1995, leading up to the 3rd Annual Meeting of the IHMF in November 1995 when recommendations on specific issues affecting the management of herpesvirus infections were debated.

The seventh IHMF workshop was held on 3-4 April 1995 to discuss latency and its implications for the clinical management of herpesviruses. All herpesviruses can exist in a latent state, but the implications of reactivation depend on the virus involved and the immune status of the host. Presentations highlighted populations at greatest risk of the consequences of latent herpesvirus infections and reviewed the strategies that have been implemented to reduce these risks. The aim of the seventh workshop was to improve understanding of the nature of latent herpesvirus infections so recommendations could be developed to limit the consequences for the affected individual in terms of discomfort and disease and for the population as a whole in terms of transmission.

The draft recommendations from the workshop were discussed at the 3rd Annual Meeting of the IHMF which took place on 17-19 November 1995. Following the Annual Meeting it was decided that the workshop topics were best illustrated in two separate Management Strategies publications entitled Genital and Orofacial Herpes Simplex Virus Infections – Clinical Implications of Latency and The Increasing Importance of Cytomegalovirus, Epstein-Barr Virus and the Human Herpesviruses Types 6, 7 and 8.

The editors would like to thank all the participants at the 3rd Annual Meeting for their contribution and especially the Co-Chairs of the working groups.

This series of monographs is jointly sponsored by the University of Alabama School of Medicine, Division of Continuing Medical Education and the IHMF. This publication is CME accredited for American and Canadian physicians (see inside back cover for details).
Objectives

The information contained in this publication should enable the physician to:

- Appreciate the worldwide seroprevalence of cytomegalovirus, Epstein-Barr virus (EBV) and human herpesviruses types 6, 7 and 8
- Recognize populations at greatest risk of infection and strategies for its prevention and/or control
- Understand the concerns for EBV-associated disease in different areas of the world
- Expand their knowledge of the most recently discovered human herpesviruses (types 6, 7 and 8) and the increasing number of diseases associated with them

Target Audience

The information contained in *The Increasing Importance of Cytomegalovirus, Epstein-Barr Virus and the Human Herpesviruses Types 6, 7 and 8* is aimed at physicians, healthcare workers and other individuals involved in the management of herpesvirus infections.
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management strategies in herpes
Herpesviruses - A Continuing Challenge

Herpesviruses are extremely common in nature and around 100 have been at least partially characterized from a variety of vertebrate and non-vertebrate animal species. There is evidence that an extremely long coevolution existed between herpesviruses and mammals. It is not surprising therefore that rapid societal changes and profound changes in host immunocompetence have created conditions which are conducive for previously unknown or rare manifestations of herpesvirus infections.

In the past 10 years, the above factors have led to the discovery of three additional herpesviruses, bringing the number of human herpesviruses to eight: herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus type 6 (HHV-6), human herpesvirus type 7 (HHV-7) and human herpesvirus type 8 (HHV-8); also referred to as Kaposi's sarcoma-associated herpesvirus (Figure 1).

An understanding of the natural history of herpesviruses has led to significant progress in controlling several diseases caused by them. With the addition of new members to the herpesvirus family, further progress in the management of herpesvirus infections will not only require improved drug discovery programmes, but also an understanding of the differences in pathogenesis.

During primary infection, herpesviruses establish latency, which allows the viral DNA to persist without expressing proteins that would be targets for an immune response. Intermittently, the latent genome can become activated to produce infectious virions. While the herpesviruses share aspects of molecular biology (replication, natural history and pathogenesis), they also differ extensively in important details. For instance, HSV-1 and HSV-2 infect a wide host-cell range, are recognized for their neurotropic characteristics, multiply efficiently and rapidly destroy the cells that they infect. EBV on the other hand is largely restricted to B lymphocytes but can induce malignant transformation. Herpesviruses also differ with respect to the cells in which they establish latency and in the clinical manifestations of disease with which they are associated.
Biological properties such as those described above have been used to classify the members of the family *Herpesviridae* into subgroups (Table 1). These depict evolutionary relatedness and serve a practical purpose in enabling the laboratory worker to identify and predict the properties of a new viral isolate by comparison with already established members.

Numerous, diverse and often opposing trends will determine the frequency and severity of herpesvirus infections in the future. A major factor is the growing population of immunocompromised patients. Sociological changes will also be important, although the consequences of these, such as attempts to influence sexual practices, will be difficult to predict. Other changes, such as more frequent use of early child-care facilities, will predictably lead to early acquisition of most herpesviruses, thereby decreasing the incidence of severe disease in adulthood.

New diagnostic methods have already associated HHV-6, -7 and -8 with specific diseases. Other factors that might increase herpesvirus-related morbidity include: the discovery of new diseases caused by previously known herpesviruses and resistance of herpesviruses to antiviral therapy.

It is likely that the existing herpesviruses will assume increasing importance in medicine over the next few years. Strategies for the management of infection must apply what is currently known about herpesvirus natural history and pathogenic mechanisms to newer members of the family and maintain constant vigilance for developments in the fields of diagnostic methods and intervention strategies.

<table>
<thead>
<tr>
<th>Alpha herpesviruses</th>
<th>Beta herpesviruses</th>
<th>Gamma herpesviruses</th>
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<tr>
<td>HSV-1</td>
<td>CMV</td>
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<td>HSV-2</td>
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<td>VZV</td>
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Table 1: Human herpesvirus subgroups according to the International Committee on the Taxonomy of Viruses (ICTV) classification

*management strategies in herpes*
Implications for Transmission of Cytomegalovirus

Cytomegalovirus (CMV) has the most complex natural history of all the herpesviruses. Most body tissues are infected with CMV during productive infection and the virus can be detected in saliva, urine, blood and semen. CMV is transmitted by all organ allografts, including blood, and can be found at autopsy in most tissues of the body. CMV thus has the ability to replicate in many, if not all, cell growth types in vivo. This makes it unlikely that CMV has a single site of latency, although possible candidates include lymphocytes and monocytes. The blood-brain barrier is easily crossed by these cells and is also a potential site for the establishment of latency.

Although a great deal has been learned about CMV, it remains the most important cause of congenital infection in the USA, a significant cause of transfusion-acquired infection and a frequent cause of morbidity and mortality among organ transplant recipients and other immunocompromised patients.

CMV infection is widespread with 40–100% of the world’s adult population being seropositive. Sources of virus include oropharyngeal secretions, urine, cervical and vaginal excretions, semen, breast-milk, tears, faeces and blood. In the healthy host, primary infection is usually asymptomatic with 95% of adults who seroconvert having no recollection of any illness. CMV disease is generally associated with either immaturity of the immune system or immunodeficiency.

The immunocompromised host represents a large proportion of those at greatest risk of reactivation of latent CMV but a comprehensive discussion of the manifestations of CMV disease and its management in this broad patient group is too large a subject for the current chapter and will form the basis of a separate Management Strategies publication in 1997. Aspects of CMV management in the immunocompromised individual with HIV are discussed in a previous Management Strategies publication (Herpesvirus and HIV Infections – Co-Factors and Opportunistic Infections). This chapter will review the implications for transmission of CMV to seronegative individuals, including pregnant women, day-care workers and transplant and transfusion recipients.

Person-to-Person Transmission

The epidemiology of CMV varies widely in different areas of the world and between different age and socioeconomic groups (Figure 1). In general, those of higher socioeconomic status have a lower seroprevalence and acquire infection later in life.

Studies conducted at day-care centres in the USA have shown that in the first year of life, approximately 10% of children shed CMV.

Figure 1: Age-related seroepidemiology of CMV infection

- USA
- Low income
- General
- Venereal disease
- High income
- Nuns
- Tanzania
This figure increases significantly to approximately 80% by the time these children have reached 12-18 months of age (Figure 2), suggesting that the children are transmitting the virus to each other by close contact. In most developed countries, infection rates increase slowly until the age of entry into school at which time they rise more rapidly. It is estimated that 40-80% of children are infected before puberty in developed countries. In other areas of the world 90-100% of the population may be infected during childhood.

**Congenital CMV infection**

Person-to-person transmission of CMV in the healthy population is generally of no clinical consequence except for the risk of congenitally acquired CMV infection. Congenital infection can be observed either after a primary infection or recurrence of a previous infection, but the probability of transmission is higher and the outcome is generally more severe if the congenital infection follows a primary maternal infection during pregnancy (Figure 3).6

**Maternal factors**

The proportion of babies who are affected by congenital CMV infection depends on a number of factors, including antibody status of the mother, breast-feeding and age. For reasons that are not known, shedding of CMV in the genital tract decreases steadily with age from up to 30% in women in their mid-teens to undetectable levels in women over 30 years old.7
There is considerable evidence that CMV-specific antibodies are important in protecting against CMV disease. In CMV-seronegative women who become infected during pregnancy, intra-uterine transmission occurs in 35–40% of cases, whereas it occurs in only 0.2–2% of women previously infected with CMV. Newborns infected after reactivation of maternal CMV infection rarely have clinically apparent disease or severe sequelae, while congenital infection after a primary maternal CMV infection during pregnancy, may result in damage to the fetus with long-term sequelae.

**Routes of transmission**

There are three routes of CMV transmission from mother to child and it is estimated that about 10% of all babies born in the USA acquire CMV through one of these routes:

- Transplacental
- Infected secretions in birth canal
- Breast-milk

Between 0.2% and 2.2% of live-born infants are infected in utero. Another 8–60% become infected perinatally as a result of infected cervical secretions in the birth canal or from breast-milk. Intra-uterine transmission of CMV can occur whether a mother has prior immunity or acquires CMV for the first time during pregnancy. The degree of protection afforded to an infected infant by the presence of antibody in the mother before conception is uncertain. It is possible for a woman seropositive for one strain of CMV to become infected with another strain of CMV during pregnancy. In post-partum women, the breast is the most common site of reactivation. Thirty per cent or more of seropositive women can intermittently excrete CMV into breast-milk during the first year after delivery.

**Populations at risk**

Subclinical shedding of CMV can provide a reservoir for transmission. Virus excretion in urine and saliva can persist for years after congenitally, perinatally and early postnatally acquired CMV infections (Figure 4); a fact that has important implications for children looked after in day-care centres. These children are excellent vectors for the transmission of CMV because of the type of contact they have with other members of society and the duration for which they are shedding virus.

**Parents**

There is a relationship between parental acquisition of CMV infection, secretion of virus by their child and the age of the child; age determining the type of contact a parent has with their child and the amount of contact with bodily secretions.
Parental seroconversion rates in families who have children attending day-care centres are approximately 20% per year. This increases to approximately 30% if the child is shedding virus and up to 40% if the child is also under 18 months of age.

A control group of parents whose children were not attending day-care centres showed no evidence of infection and therefore did not seroconvert. This has important implications for CMV-seronegative mothers with a child at a day-care centre who are planning or expecting another child because of the risks of acquisition of primary CMV infection and congenital CMV infection.

Day-care workers
Day-care workers also have an increased risk of acquiring CMV infection compared with the expected annual seroconversion rate of 2% per year (Table 1). This again has implications for women of child-bearing age working in this environment.

Hospital workers
Little evidence has been found of a greater risk of acquisition of CMV in hospital workers. Hygiene is at a standard that could not be achieved in a day-care centre or in the home, and of the proportion of patients hospitalized the number likely to be shedding CMV is small.

Sexually active individuals
Sexual transmission of CMV has been suggested as the explanation for the increase in prevalence of CMV infection with increasing age after puberty. Recurrences are associated with intermittent shedding of CMV from many sites and a number of findings indicate that CMV can be transmitted during sexual contact. In addition, increased seroprevalence and excretion of virus have been found among women attending sexually transmitted disease clinics.

<table>
<thead>
<tr>
<th>Study</th>
<th>Conversion rate/year (%)</th>
<th>Risk ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adler</td>
<td>11.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Murph et al</td>
<td>7.9</td>
<td>4.0</td>
</tr>
<tr>
<td>Pass et al</td>
<td>20.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

*Assumes expected rate of seroconversion of 2%/year

Table 1: Transmission of CMV to day-care workers – summary of cohort studies

Strategies for the prevention of transmission
Because the majority of primary infections with CMV are subclinical and because of the high seroprevalence in most populations, little can be done at present to prevent acquisition. Thorough cleansing after contact with bodily secretions of young children is recommended, but it is not known if this is successful or if the level of hygiene required is readily achievable in the home.
In the future, a vaccine may be available and, although it may not prevent transplacental infection, it might reduce the sequelae observed in infants born to mothers with primary infections during pregnancy to the rate observed in seropositive mothers. In the USA, this would reduce the number of children with morbidity and mortality as a result of CMV infection from approximately 8000 to 1000 per year.

Transfusion-Associated Transmission

Populations at risk

Patients at greatest risk of transfusion-acquired CMV infection are those with an immature immune system or those who are immunosuppressed. For CMV-seropositive patients receiving blood from seropositive donors, the risk of CMV infection varies from 5% to 67%, with an average of 14%. Approximately 30% of infected patients become symptomatic. In these patients, reinfection with donor CMV may cause more severe disease than reactivation of the recipients’ latent virus.13

Infection rates with transfusion of infants are only slightly increased over those in older individuals, but with primary infection in premature babies CMV-induced disease is much more common. Between 50% and 90% of the seronegative infants develop various forms of CMV-induced diseases and mortality rates can range as high as 40%.13

Exactly which blood components actually harbour infectious virus in seropositive individuals and can transfer it to the recipient is not known, but development of both primary and recurrent CMV infection post-transfusion depends on the amount of blood or blood products transfused and the number of seropositive donors. A risk of 3–4% per unit of unscreened blood has been estimated.14

Strategies for the prevention of transmission

Screening

The transmission of CMV by blood transfusions can pose a serious risk to certain immunocompromised patients and, therefore, a sensitive method is needed for detection of the virus when screening blood. However, there have been inconsistencies with isolation of CMV from the blood of healthy donors, with some but not all studies, being able to isolate virus.15,16 There are also inconsistencies with CMV detection by the polymerase chain reaction (PCR). One study detected CMV DNA in 100% of seropositive donors as well as 50% of seronegatives.17 However, other authors failed to confirm these data.18

Ideally, susceptible individuals undergoing blood transfusion should be given blood from CMV-seronegative donors to reduce the incidence of infection. However, this cannot be done routinely in settings where the prevalence of CMV infection is very high due to an obvious shortage of donors and the costs involved. In this situation it is advisable to use blood components from seronegative donors to protect those at greatest risk from infection, such as premature infants and those who will require frequent or large volume transfusions (Table 2).
Chapter 2

Management strategies in herpes

**Table 2: Populations at risk of severe disease from transfusion-associated CMV infection**

- CMV-seronegative pregnant women
- Premature infants
- Allogeneic bone marrow transplant recipients
- Individuals with advanced HIV disease
- CMV-seronegative patients receiving multiple transfusions
- CMV-seronegative cancer patients receiving high-dose chemotherapy
- CMV-seronegative burn patients
- Patients with severe combined immunodeficiency

### Treatment of blood products

Considerable literature has accumulated indicating that leucocytes present in allogeneic cellular blood components intended for transfusion are vectors for infectious agents such as CMV. Although the threshold leucocyte number at which disease manifests is still to be determined, leucocyte-deprived blood is thought to be relatively safe.

Other methods include de-glycerolized frozen red cells and, currently under investigation, inactivation with photo-active compounds. A further strategy in the prevention of blood-borne infections in general must be to restrict transfusions only to those indications that are essential for the survival of the patient.

### Vaccination

There is considerable evidence that antibodies provide protection from CMV disease. For instance, about 85% of transplant patients with primary infection become symptomatic compared with only 20–40% of patients suffering recurrent infection. In addition, studies in mice have illustrated the importance of neutralizing antibodies in protecting mice from lethal challenge.

There are several different strains of CMV, however, which present different epitopes and immune individuals may become re-infected with a different strain. A vaccine would therefore need to be broadly immunogenic because of the genetic disparity among different CMV strains.

If such a vaccine were to become available it would prove beneficial for a considerable number of patients, including CMV-seronegative patients undergoing transplantation, HIV-positive individuals to prevent re-infection with another CMV strain and susceptible females wishing to start a family.
Summary and Recommendations

The following guidelines for the management of cytomegalovirus infections (in terms of transmission) and summaries of the clinical implications for Epstein-Barr virus and the more recently discovered human herpesviruses types 6, 7 and 8 have been prepared following the IHMF workshop of 3-4 April 1995 and the 3rd Annual Meeting of the IHMF, held in Istanbul, 17-20 November 1995.

Implications for Transmission of Cytomegalovirus

While cytomegalovirus (CMV) infection is generally without consequences in the immunocompetent individual, it can be particularly severe in the neonate and in the immunocompromised host.

Person-to-person transmission

Newborns infected with CMV usually acquire the virus as a result of infected cervical secretions in the birth canal or in the breast-milk. Because these children can then secrete virus for periods of up to 5 years, a particularly high rate of transmission of CMV has been found between children at day-care centres. If a mother of one of these children is seronegative and plans to have another child there is an increased risk of her seroconverting during pregnancy and of the neonate acquiring congenital CMV infection. Pregnant day-care staff are also at risk of transmission.

Little can be done at present to reduce these risks other than educating physicians, parents and day-care staff about the possibility of CMV infection. Physicians need to be able to counsel women about the sources of CMV infection and the risk to the fetus if the mother acquires primary CMV infection during pregnancy. It is recommended that women should be able to obtain up-to-date information from their physician on the risks of CMV infection during pregnancy. Such information may include: sources of maternal CMV infection, risk to the fetus and means of prevention. Institutions such as day-care centres and schools should also be provided with relevant information to develop policies, e.g. written guidelines on thorough cleansing after contact with body fluids, which can be followed in an attempt to limit transmission within these higher risk environments. Screening of all pregnant women and day-care staff is not recommended and probably not feasible. Serological testing accompanied by appropriate counselling may be useful for individual patients concerned about their risk of acquiring CMV infection.

In the future a CMV vaccine may be offered to women at risk. Until such a vaccine becomes available, educational efforts and stricter hygiene are considered the best approaches to limiting transmission. It is worth noting, however, that these approaches lack proven efficacy and do not address the possibility of congenital infection from reactivation or re-infection in women who have been infected with CMV in the past.

Transfusion-associated transmission

The transmission of CMV by blood transfusion can have serious consequences in susceptible individuals and is a frequent cause of disease. It is therefore desirable to
limit the use of blood transfusions in these patients wherever possible. To protect patients at greatest risk of infection, e.g. premature babies and seronegative individuals, it is advisable to use blood components from seronegative donors. However, this is not always possible where a large proportion of the population is infected with CMV and because of inconsistencies in screening. Other strategies to reduce transmission via blood transfusion are therefore being evaluated such as those which involve treatment of blood products.

The removal of blood leucocytes by filtration has been used with some success, although the efficacy of this method may not be consistent for all blood products and published data on the use of blood filtration to prevent transfusion-associated transmission of CMV in immunocompromised patients are limited.

Implications for Reactivation and Transmission of Epstein-Barr Virus

Unlike the other herpesviruses, Epstein-Barr virus (EBV) is linked to a number of lymphoproliferative diseases which are associated with the ability of the virus to immortalize B lymphocytes.

Malignancies associated with reactivation of the virus from a latent state include nasopharyngeal carcinoma (NPC) and most of the lymphomas found in immunocompromised patients.

In certain areas of the world NPC is endemic and responsible for a large proportion of the cancer deaths. However, the association of EBV reactivation with this carcinoma is so constant that routine serological screening for increased antibody levels can be used for diagnostic purposes, thus allowing early intervention. Other factors determining at-risk individuals include genetic and environmental factors.

Although a vaccine is not currently available, it is conceivable that several malignant diseases associated with EBV may be avoided if primary infection could be prevented. However, because the majority of populations are infected early in life, it may be difficult to design appropriate trials. In the future a therapeutic vaccine could be considered for use in association with serological screening for NPC.

Primary EBV infection in the adolescent or adult immunocompetent host varies in its duration and severity. Although antiviral drugs such as aciclovir and ganciclovir are effective against oropharyngeal EBV replication they appear to have only marginal clinical benefit on the severity or duration of primary EBV disease. In addition, clinical symptoms associated with EBV reactivation generally become evident a long time after primary infection. These factors have restricted the use of antiviral therapy for the treatment of EBV.

Clinical Implications for Human Herpesviruses Types 6, 7 and 8

With the introduction of more sensitive and rapid diagnostic methods the three latest members of the human herpesvirus family have all been linked to a disease. Human
herpesvirus type 6 and 7 (HHV-6 and HHV-7) appear widespread in the population and have been shown to be aetiological agents in the development of a usually mild childhood illness, exanthem subitum. In the immunocompromised the situation is less clear but reactivation appears to be associated with the degree of immunosuppression as a higher frequency of HHV-6 viraemia is observed after bone marrow compared with renal transplantation.

Human herpesvirus type 8 (HHV-8) was originally isolated from AIDS-associated Kaposi’s sarcoma tissue and a large body of evidence now suggests that HHV-8 has a causal role in this disease. HHV-8 does not appear to be widespread in the general population and it is possible that it may be sexually transmitted as AIDS-associated Kaposi’s sarcoma is primarily found in homosexual men. Whether this virus plays a role in the pathogenesis of other diseases in the immunocompetent or immunocompromised host has not been established.

Information on the epidemiology of these relatively recently discovered herpesviruses is accruing rapidly but is a long way from being complete and will require an understanding of the pathogenesis of disease in different patient groups. As yet there are no set guidelines for management of patients with these infections, but as HHV-6, HHV-7 and HHV-8 become causally linked to an increasing number of different diseases there is clearly a need for clinical trials to be conducted on the effectiveness of new and existing antivirals.
References

CHAPTER 1

CHAPTER 2
management strategies in herpes


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Cytomegalovirus (CMV) infections are common and usually asymptomatic in otherwise healthy children and adults (13). CMV has a worldwide distribution, infecting between 40% and 90% of adults, leading to lifelong latent infection (1).

However, for recipients of solid-organ or hematopoetic-cell allografts and individuals with advanced AIDS, CMV is a well-known cause of serious morbidity and sometimes fatal infections (14). Recurrent CMV disease has been reported to occur in 6 to 59% of solid-organ transplant recipients (5). The risk factors cited for the development of CMV disease in transplant recipients are graft rejection, antilymphocyte therapy, and high-dose corticosteroids (11). Patients who develop CMV infections incur costs associated with diagnostics, hospitalization, and multiple physician visits. For a transplant patient who has been diagnosed with severe CMV disease, these costs have been estimated at $14,000 (21).

Following the implementation of universal rubella immunization programs, CMV has become a common cause of intrauterine infection, with between 0.3 and 2.4% of neonates becoming infected in different countries (19). In some countries, congenital CMV infection may affect more children than do other, better-known childhood conditions, such as Down syndrome, fetal alcohol syndrome, and spina bifida (2, 17). Data from the United States of America have led to an estimate that 8,000 newborns have health problems each year as a result of congenital CMV infection (3). Analysis conducted in the early 1990s revealed that the estimated costs to the U.S. healthcare system associated with congenital CMV infection were approximately $1.9 billion annually (6), with a cost per affected child of over $300,000 (7).

In a recent review of the priorities for vaccine development, CMV was ranked in the highest of five tiers by the Institute of Medicine in the United States as a potentially cost-saving vaccine target (21). The ranking was based on the economic cost and years of disability that would be avoided and the years of life saved by an effective vaccine.

There are several promising candidate vaccines against CMV in various stages of preclinical and clinical testing, including recombinant vaccines, live attenuated vaccines, and chimeric vaccines (18). Progress in the development of vaccines against CMV encouraged us to examine the seroepidemiology of CMV in Australia.

This study therefore aimed to determine the seroprevalence of CMV in Australia in relation to age and sex. These results of this study will inform future vaccination strategies and public policy.

MATERIALS AND METHODS

Serum samples. A total of 3,593 serum samples were tested for CMV seroprevalence as part of the National Centre for Immunisation Research and Surveillance of Vaccine Preventable Diseases (NCIRS) serosurveillance program. Samples were collected in 2002 from 37 major diagnostic laboratories throughout Australia that agreed to participate in the program. These laboratories supplied remnant sera from samples that had been submitted for serological testing and would otherwise have been discarded. Sera from subjects who were either immunocompromised, had received multiple transfusions in the past 3 months, or were known to be infected with human immunodeficiency virus were excluded (8). Sera were identified at the referring laboratory by the sex of the subject, age or date of birth, residential postcode, date of collection, and a unique identifier to ensure that only one sample from any subject was tested. These identifiers were removed from the samples before testing, and the samples were coded by date of collection, state/territory of origin, and referring laboratory.
Study population. Serum samples were collected from people between 1 and 59 years of age and stratified into the following age groups: 1 to 2, 3 to 4, 5 to 9, 10 to 14, 15 to 19, 20 to 24, 25 to 29, 30 to 39, 40 to 49, and 50 to 59 years. Serum samples were not available for infants less than 1 year of age. Approximately equal numbers of males and females were tested. Within each age group, states and territories were sampled in proportion to their population size. Prevalence was calculated separately for each age group and for Australia as a whole by weighting the prevalence estimates for each age group by the age distribution of the 2002 Australian population. The CMV prevalence for the group aged 60 years and above was assumed to be the same as for the group aged 50 to 59 years. Sample sizes were calculated to achieve a 95% confidence interval (CI) of approximately 5% for each age group.

Serological testing. Serum samples were tested for CMV-specific immunoglobulin G (IgG) using a CMV IgG enzyme-labeled antigen test (Medac, Hamburg, Germany), and the results were interpreted according to the manufacturer’s instructions. Samples with optical densities 10% or more below the cutoff were recorded as negative, those with optical densities between 10% below and 10% above the cutoff were equivocal, and all others were positive. If the sample’s absorbance was within 10% of the cutoff level, the sample was retested and classified according to the retest result.

Statistical methods and ethical approval. The percentages of individuals with positive, negative, and equivocal results were determined for each age group and sex. Epi-Info version 3.3.2 was used for the analysis and comparison of serostatuses among age groups. Ninety-five-percent confidence intervals were calculated where appropriate, and P values of <0.05 were considered statistically significant. Ethics approval was obtained from the Human Research Ethics Committee of the Western Sydney Area Health Service.

RESULTS

Age-specific seroprevalence. The seroprevalence of CMV in the population tested was 57% (95% CI, 55.2 to 58.6%), with an overall association identified between seroprevalence and increasing age (Table 1). For the groups of children aged 1 to 2 and 3 to 4 years, the recorded seroprevalences were 38% (95% CI, 32.2 to 44.3%) and 39% (95% CI, 34.0 to 44.5%), respectively. Seroprevalence continued to rise with age from 50% (95% CI, 48.5 to 58.7%) in 20- to 24-year-olds to 79% (95% CI, 72.7 to 84.7%) in 50- to 59-year-olds (Fig. 1).

Gender-specific seroprevalence. There was little overall difference in seroprevalence between males and females (51% and 54% seropositive, respectively; P = 0.09). Although the percentages that were seropositive did not differ between the sexes overall, they did differ by age group (Fig. 1). In the younger age groups, slightly greater percentages of males than females (Fig. 2) were seropositive, and in the adult age groups, the reverse was true. For females (Fig. 3), the levels of CMV seroprevalence increased substantially between the group aged 30 to 34 years and that aged 35 to 39 years (56% and 79% seropositive, respectively; P value = 0.0005).

DISCUSSION

The population-weighted rate of CMV seropositivity in subjects between 1 and 59 years of age was found to be 57%. This rate was found to increase with age, so that by 20 years of age, 53% of the tested population was seropositive. For women, a significant rise in CMV seroprevalence occurred between the group aged 30 to 34 years and that aged 35 to 39 years. This may be explained by motherhood and the fact that women in
these age groups have close contact with young children, who may act as a vehicle for transmission.

A study conducted in Australia by Kelly et al. showed that anonymous opportunistic testing of sera provides estimates of immunity that are comparable with those of random population-based surveys of some vaccine-preventable diseases (12). However, the lack of detailed information about participants in opportunistic serosurveys means that it is not possible to identify or control for various potential biases. A number of measures were implemented to ensure that selection bias was reduced. These included enrollment of major laboratories that represented both regional and rural areas and the use of sera that were collected from outpatients rather than hospitalized patients.

These are, to our knowledge, the first published results on the national seroepidemiology status of CMV in Australia. Previously published studies have examined only select subpopulations. The first study (10), published in 1968, examined stored sera from children and adults living in only one Australian state. The sera were collected from a range of sources, including hospitalized children and their parents, blood donors, and patients attending antenatal clinics. The study concluded that infection appeared to be acquired with increasing frequency early in adult life. However, as these findings were not published by age group, it was hard to make a distinction about when the increase occurred (10). A more recent Australian study (15) examined the baseline CMV IgG seroprevalence by the use of blood donor sera. The study again was limited to one state in Australia. This comparative study found that 35% of subjects less than 20 years of age were seropositive for CMV IgG, whereas our study identified rates of seroprevalence in children and teenagers ranging from 39% in 3- to 4-year-olds to 50% in 15- to 19-year-olds. For people over the age of 50 years, the comparative study found that two-thirds (72%) were seropositive, a finding that was consistent with our study results.

A review of CMV seroprevalence studies conducted around the world reveals that residents of developing countries have higher rates of CMV seropositivity than those of developed countries (4). In general, CMV is acquired earlier in life in developing countries and among the lower socioeconomic strata of developed countries (20). In some African nations, seropositivity rates reach 80 to 90% by 10 years of age (19); in comparison, in the United States and Great Britain, certain subgroups of children have CMV seropositivity rates below 20% at 15 years (19).

In contrast with the vaccination rates needed for the elimination of measles (93%), mumps (93%), and rubella (92%), studies have shown that CMV requires a much lower rate of vaccination (60%). This is related to the fact that CMV has a low force of infection and a relatively low basic reproductive number (R0) of 2.5 (9). R0 refers to the number of secondary infections caused by the introduction of a single infectious case into a completely susceptible population and is affected by several factors, including the duration of infectivity of affected patients, their contact with susceptible people, and the infectious nature of the organism.

The finding that the highest levels of CMV exposure occur in the first few years of life (Table 1) suggests that, for a universal vaccination program to have maximal impact, the vaccine would need to be delivered to infants and have a long duration of protective efficacy. If the duration was only brief, a vaccine given just before pregnancy would be advised, or, if the vaccine had a medium duration of efficacy (5 years), regular boosters could be given throughout childhoodbearings. Concerns are raised with strategies based on targeting women prior to or during pregnancy. These include the cost and time related to the establishment of screening programs to identify CMV-specific antibody in pregnant women. Also, past experience with targeted programs for pregnant women in Australia has shown low rates of vaccine uptake, even in situations of high risk such as that with carriers of hepatitis B (16). It should be noted that cohorts of immunized children would have to reach childbearing age before CMV disease could be completely brought under control. This is due to the persistent nature of CMV disease and its tendency to reactivate periodically (9). As with other childhood diseases, CMV has the potential to infect children prior to the scheduled age of vaccination; therefore, successive generations would need to be immunized before the disease could be eradicated (9).

The potential benefits of a CMV vaccine would include reduced transmission to pregnant women and less CMV disease due to primary infection or reactivation in organ transplant recipients and the immunosuppressed (9). Stratton et al. have suggested that if a vaccine program which had 100% efficacy and 100% uptake were implemented today, the annualized present value of the quality-adjusted life-years gained would be 70,000 (21). They went on to suggest that the vast majority of these quality-adjusted life-years would be attributed to the lack of the long-term sequelae now experienced by infants who acquire congenital CMV infections (21).

A decision to introduce vaccination will depend on the epidemiology of infection and on the safety of the vaccine. Studies such as this one provide valuable information that can be used to examine the epidemiology of infection in the community and to focus the administration of a future vaccine on populations at high risk of the disease.

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REFERENCES


What is Polio?

POLIO

Polio, or more properly poliomyelitis, was one of the most feared and studied diseases of the first half of the 20th Century. Though the Salk and later the Sabin vaccines have essentially eliminated the disease in developed countries, many mysteries regarding polio remain. This is probably due to the fact that as polio epidemics ended in North America and Europe, research on the disease also came to an abrupt halt. Thus, as LaForce (1983) noted, knowledge about the epidemiology and pathology of polio is essentially frozen at a mid-1950s level.

In the paragraphs that follow, I will define the disease of polio and explore some of the mysteries that still surround it. Other sections of this site document polio's history, describe its late effects, and provide excerpts from narrative accounts of various aspects of the polio experience.

Poliomyelitis is an "inflammation of the gray matter of the spinal cord (Taber, 1970, p. P-77). Though the word "poliomyelitis" sounds complicated and impressive, it was formed by putting together the Greek words for the site of the disease - polios, meaning gray, myelos, meaning marrow, and adding the English suffix, itis, meaning inflammation. It has gone by many names including infantile paralysis, Heine-Medin's Disease, debility of the lower extremities, and spinal paralytic paralysis. In common usage, the term poliomyelitis is abbreviated to polio.

Polio is caused by a virus which results in an acute infection. However, contrary to what is commonly believed, the virus did not typically result in paralysis. Rather, the majority of infected individuals experienced only mild respiratory or gastrointestinal symptoms, often accompanied by fever, headache, and muscle stiffness. These symptoms lasted only a few days, and many had such mild cases that they did not even realize they were ill. Therefore, they often continued on with there daily routines, attending school or work, and exposing many others to the polio virus. This fact helps explain the reports of many polio survivors that they were the only ones in their family, neighborhood, or community to have had polio. In actuality, there could have been many individuals with whom they came into contact who had the minor illness, as non-paralytic polio was often called.

Only in a small number of cases did the virus penetrate the central nervous system, causing the major illness, or "true polio." In these cases, neurons (nerve cells) in the anterior horns of the spinal cord and the lower brain were affected, resulting in "tightness in the neck, back, and hamstring muscles as well as varying degrees of muscle weakness, as paralysis sets in" (Owen, 1990, p. 211). Though there was never a cure for polio, most who contracted it experienced improvement in muscle strength and control after the acute infection subsided. In some cases, however, motor neurons were left severely damaged or completely destroyed, resulting in permanent weakness or paralysis, most commonly to the lower extremities (Headley, 1995).

There are actually three separate strains or immunologic types of the disease: Type I (Brunhide), Type II (Lansing), and Type III (Leon). Most epidemics, at least in the United States, were the result of Type I virus (Nathanson and Martin, 1979).

The means by which the polio virus enters the central nervous system is still not definitely known. (Taber, 1970). However, it is interesting to note that relatively recent data from third-world nations suggests that when those experiencing acute polio are given injections such as
DPT immunizations, antibiotics, antimalarials, or antipyretics, the injected limb develops paralysis within one week (LaForce, 1983).

Though this connection between injections and paralysis has repeatedly been demonstrated, "the mechanism by which this phenomenon occurs is not well understood. (However) the best evidence to date suggests the trauma initiates a reflex dilation of blood vessels at the corresponding spinal cord level and facilitates entry of the virus" (LaForce, 1983, p. 30).

Other factors thought to predispose individuals to the major disease were pregnancy and tonsillectomy, as well as other nose and throat procedures. Why these conditions were likely to increase the risk of the disease entering the central nervous system remains unknown.

There is also still some mystery surrounding the exact means by which the polio virus is transmitted. As Smith (1990) noted in her history of the development of the Salk vaccine, "Nobody has ever completely settled the question of how polio is spread, though the best evidence suggests the virus is excreted in the stool and passed through hand-to-hand contact . . . " (p. 36).

The belief that the polio virus is spread by contact with the feces of an already infected person has been offered as an explanation for the increased incidence of polio in developed countries such as the United States during the 20th Century. According to this theory, before the advent of modern sewage treatment plants and other improvements in public sanitation, virtually all individuals were exposed to the polio virus early in their lives when they were at least partially protected by maternal antibodies. Thus, they developed mild, non-paralytic infections, probably during infancy, which provided them with lifelong immunity. However, with better sanitation, both these early infections as well as the likelihood of receiving antibody protection decreased, resulting in greater susceptibility to paralytic polio. Thus, in the words of Smith:

Put simply, paralytic polio was an inadvertent by-product of modern sanitary conditions. When people were no longer in contact with the open sewers and privies that had once exposed them to the polio virus in very early infancy when paralysis rarely occurs, the disease changed from an endemic condition so mild that no one knew of its existence to a seemingly new epidemic threat of mysterious origins and terrifyingly unknown scope (p. 23).

This central theory regarding the spread of polio is supported, at least to some extent, by experiences in third world countries. During World War II, for instance, U.S. and British troops stationed in undeveloped countries were much more likely to contract polio than native peoples, who apparently had already developed immunity (Paul, 1971). Even in the 1970’s, when individuals from developed countries came into contact with those from a country without a modern sanitation system, the incidence of paralytic polio was about twenty times greater for those from the developed country (Nathanson and Martin, 1979).

The above explanation for the transmission of polio is generally accepted and seems quite logical. However, the incidence of the disease in the United States during the epidemic years was very irregular, not only from year to year, but from area to area, apparently showing no relation to improvements in sewage treatment. The actual reason for this variation remains another of the polio mysteries. However, it has been suggested that this variability was possibly due to increased virulence of certain virus strains or the presence of environmental conditions that enhanced the disease’s transmission (Nathanson and Martin, 1979).

Regarding the second factor of environmental conditions, it is well documented that polio was primarily a disease of the summer months. Readers old enough to recall the epidemic years of the 1940's and 50's probably have vivid recollections of community swimming pools and other public areas being closed during the "dog days" of July and August. Perhaps this was an appropriate precaution. The disease occurred thirty-five times more frequently in August than in April (the month of lowest incidence). Though no one really knows why this was the case, it does not appear that seasonal variation in interpersonal contacts is a
sufficient explanation. Rather, it is more likely that warm, moist weather favored transmission of the disease, as Bradshaw (1989) and others have noted. This is, however, just a hypothesis. The actual reason for the seemingly random appearance of polio epidemics remains a mystery to this day. Since the disease is all but dead, it seems unlikely that all of polio's mysteries will ever be solved.
A population-based description of glioblastoma multiforme in Los Angeles County, 1974-1999

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KEYWORDS
glioblastoma multiforme • epidemiology • incidence • race • outcomes

ABSTRACT

BACKGROUND
There have been reports that the incidence rates of brain tumors have increased over the past few decades, but most have considered all brain tumors together. The authors analyzed the pattern of glioblastoma multiforme (GBM) occurrence in Los Angeles County, California to shed light on the incidence and descriptive epidemiology of this type of brain tumor.

METHODS
Data were obtained from the Los Angeles County Cancer Surveillance Program. Incidence rates were analyzed by gender, race, age at diagnosis, period of diagnosis (1974-1981, 1982-1988, or 1989-1999), and socioeconomic
status (SES). In addition, data were stratified according to anatomic subsite. A multivariate model describing changes in rates by each of these variables was constructed.

RESULTS
Age-specific incidence rates (ASIR) rose sharply after age 30 years. The peak ASIR was at age 70-74 years in males and at age 75-79 years in females. The age-adjusted incidence rate (AAIR) of GBM increased from 1974 to 1999 by an estimated 2.4% per year among males and 2.8% per year among females. Overall, males had a 60% increased risk of brain tumors compared with females. Males had a higher incidence of GBM compared with females at each anatomic subsite except the posterior fossa. The largest male:female ratio occurred in the occipital lobes. Non-Latino whites had the highest incidence rates (2.5 per 100,000) followed by Latino whites (1.8 per 100,000), and blacks (1.5 per 100,000). After 1989, compared with the period before magnetic resonance imaging (MRI) was available, there was an increase in GBM incidence rates among those with of higher SES that was most pronounced in females. The incidence of GBM was highest for frontal lobe tumors and for tumors that involved two or more lobes (overlapping tumors), followed by tumors in the temporal and parietal lobes. In the multivariate analysis, year of diagnosis, SES, gender, race (Latino but not black), site, and age at diagnosis all were important predictors of incidence rate.

CONCLUSIONS
GBM incidence increased in Los Angeles County over the last 30 years and especially after 1989, suggesting that the introduction of MRI may have contributed to the increase. Individuals older than age 65 years experienced the greatest increase in incidence over time. Older age, male gender, higher SES, and non-Latino white race increased the risk of GBM. Previously unreported incidence rates for GBM among Latino whites were significantly lower than among non-Latino whites but were intermediate between non-Latino whites and blacks. Cancer 2005. © 2005 American Cancer Society.
IV. PATHOPHYSIOLOGY IN ACUTE POLIO.

Knowledge of the pathophysiology of acute polio is necessary to understand the possible causes for PPS and to provide a rational basis for its management. The poliovirus is a positive single-stranded RNA enterovirus belonging to the picornavirus group. The virus is relatively small (30 nm in diameter) and the poliovirus genome is about 7400 nucleotides long. It lacks a lipid coat or capsule but has a protein coat shaped like a polyhedron with 20 faces. There are three polio viruses, numbers 1, 2, and 3, defined by the configuration of the capsid proteins. Therefore, theoretically, a person could be infected more than once.

Wild poliovirus enters the body by oral ingestion, then replicates in the lymphoid tissue of the pharynx and ileum and spreads regionally to lymphoid tissue. It is extremely infectious and usually benign. The vast majority of infected individuals (95 to 99%) remain asymptomatic or experience a self-limited illness characterized by fever, myalgia, and gastrointestinal symptoms. However, in 1 to 5% of persons, viremia may follow, with invasion of the anterior horn cells of the central nervous system (CNS). These patients usually develop a headache, stiff neck, and back pain, similar to viral meningitis. Only 1 to 2% of all those infected develop paralysis. Finally, the rate of paralysis varies with the strain of the virus and the patient's age. In children, paralysis occurs in 1/1000 cases, while in adults 1/75 develop paralysis. Asymmetric, flaccid paralysis occurs, with legs more commonly involved than arms. Severe bulbar weakness occurs in 10 to 15% of all paralytic cases. Less frequently, there is ophthalmoplegia and bladder involvement. The pathological findings of acute polio consist of inflammation of meninges and anterior horn cells, with loss of spinal and bulbar motor neurons. Less prominent findings include abnormalities in the cerebellar nuclei, reticular formation, thalamus, hypothalamus, cortical neurons, and dorsal horn.

Once the virus has invaded the CNS, neurological and functional loss occurs as anterior horn cells are lost, and thus the muscle fibers innervated by them are "orphaned". Recovery begins in weeks and reaches a plateau in 6 to 8 months. The extent of neurological and functional recovery is determined by three major factors: (1) the number of motor neurons that recover and resume their normal function, (2) the number of motor neurons that develop terminal axon sprouts to reinnervate muscle fibers left orphaned by the death of their original motor neurons, and (3) muscle hypertrophy. The phenomenon of terminal axon sprouting makes it possible for an uninvolved or recovered motor neuron to "adopt" these orphaned muscle fibers. Stålberg has shown that a motor neuron cell can adopt five to seven additional muscle fibers commonly and occasionally, as many as 20 for every muscle cell innervated originally. A single motor neuron that originally innervated 100 muscle fibers might eventually innervate 700 to 2000 fibers. As a result, the survivors of acute polio may be left with a few, significantly enlarged motor units doing the work previously performed by many units. Figures 3, 4, 5, and 6 provide a schematic illustration of this phenomenon. Both electrophysiological evidence, including single fiber and macro-EMG and morphological data support this concept.

In addition to this reinnervation, the remaining muscle fibers hypertrophy to increase the strength of the muscle group. Because this mechanism of neurophysiological compensation is so effective, a muscle can retain normal strength even after 50% of the original motor neurons have
been lost. Therefore, in some patients, manual muscle testing (MMT) may be normal when more than half the original anterior horn cells are destroyed. [29]

FIGURE 3. A normal motor unit containing the motor neuron and the muscle fibers it innervates.
**FIGURE 4.** Details of the motor unit.
FIGURE 5. Pathophysiological changes seen with acute poliomyelitis.
FIGURE 6. Reinnervation through collateral sprouting.
Enhanced Replication of Human Cytomegalovirus in Human Fibroblasts Treated with Dexamethasone

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SUMMARY

The effect of glucocorticoid hormones on the replication of human cytomegalovirus (HCMV) was studied in human embryonic lung (HEL) cells. Treatment of cells with pharmacological concentrations of adrenal glucocorticoids such as dexamethasone enhanced HCMV replication; treatment with oestrogenic or androgenic hormones did not do so. In dexamethasone-treated HEL cells there was an approximately tenfold increase in virus yield, with the virus eclipse period shortened by 1 day compared to control cultures. Treatment of cells with the hormone also enhanced plaquing efficiency of the virus by approximately tenfold. As the synthesis of virus-specific immediate early proteins and antigens was notably enhanced together with an increase of HCMV DNA synthesis, it appeared that the early stages of the HCMV replication cycle might be under hormonal control. Moreover, the data presented suggest that the hormonal enhancement of HCMV replication involves specific receptor proteins and requires the synthesis of a specific cellular mRNA(s).

INTRODUCTION

Herpes simplex virus (HSV) has a broad spectrum of pathogenicity and productive cytolitic activity in vivo and in vitro. In contrast, human cytomegalovirus (HCMV) infection is restricted to man and its replication in vitro is supported only in human diploid fibroblasts (Smith, 1959). On a molecular level, early after virus infection, HSV inhibits the synthesis of host cellular macromolecules (Ben-Porat & Kaplan, 1973). Conversely, HCMV stimulates the synthesis of cellular macromolecules and of cellular enzymes (Furukawa et al., 1975a, 1976; St. Jeor et al., 1974; Tanaka et al., 1975; Hirai et al., 1976; Estes & Huang, 1977). When this stimulation is inhibited chemically, by serum starvation or by u.v. light irradiation of host cells, HCMV replication is markedly reduced or eliminated (DeMarchi & Kaplan, 1977; Furukawa et al., 1975b). These observations suggest that the replication of HCMV is dependent on stimulated host cell functions.

Adrenal glucocorticoid hormones such as dexamethasone are known to play a role in the modulation of transcription of host cell DNA. The hormones first bind to a specific receptor protein present in cytoplasm (Yamamoto & Alberts, 1976) and then these hormone–receptor complexes bind to cellular DNA (Jensen et al., 1968; Gorski et al., 1968). This interaction has significant effects on synthesis and translation of host cell mRNA (Higgins & Gehring, 1978). Therefore, it is of interest to study whether these hormones affect the replication of HCMV in the permissive system.

In this report we describe the effect of glucocorticoid hormones on the replication of HCMV. The results clearly show that treatment of human embryonic lung fibroblasts with pharmacological concentrations of dexamethasone significantly enhances HCMV replication.
**METHODS**

*Cells and virus.* Human embryonic lung (HEL) cells, prepared from 4-month-old foetal lung by explanting minced tissue, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. HCMV-infected HEL cells were maintained with DMEM containing 5% calf serum. The Towne strain of HCMV was used in these studies. Stocks of virus were prepared by infecting confluent monolayers of HEL cells with HCMV at a multiplicity of infection (m.o.i.) of 0-01 p.f.u./cell. Three to 5 days after cytopathic effect (c.p.e.) had developed in 100% of the cells, culture media were harvested and clarified by centrifugation (1500 g for 15 min). The supernatants were collected and stored at -85 °C. Cells and virus were found to be free of mycoplasma contamination by inoculation into PPLO broth (Difco) as described previously (Stinski, 1976).

*Treatment of HEL cells with corticosteroid hormones.* Stock solutions of hormones were prepared in 99.5% ethanol. The hormones, when present, were given 24 h before virus infection. The cells were washed three times with Hanks' balanced salt solution (HBSS), virus was inoculated and maintenance medium or agarose overlay medium (for plaque assay) containing hormones was added. Control cultures were similarly treated with DMEM containing amounts of ethanol equivalent to those in the hormone-treated cultures. All hormones used in these experiments were obtained from Sigma.

*Titrations of infectious virus.* Semi-confluent monolayers of HEL cells grown in 1 oz prescription bottles were infected with HCMV. After a 90 min adsorption period, the cells were washed twice with HBSS and maintenance medium was added. At various intervals post-infection, the total amount of infectious virus was measured after the cells had been disrupted by freezing and thawing once, and by sonication (Branson sonifier) for 30 s. The infectious virus titre was determined on HEL cells using the plaque assay described by Wentworth & French (1970).

*DNA labelling and analysis.* The method used for DNA analysis was, in general, that described by Crouch & Rapp (1972). HEL cells grown in 4 oz prescription bottles were infected with HCMV at an m.o.i. of 1. After 90 min adsorption, the cells were washed with HBSS and maintenance medium was added. The cultures were labelled with [Me-3H]thymidine (5 μCi/ml; sp. act. 101 Ci/mmol; New England Nuclear) at 24 h post-infection and incubated for an additional 24 h. The cells were rinsed three times with ice-cold TES buffer (10 mM-Tris-HCl pH 7.4, 10 mM-EDTA, 50 mM-NaCl), lysed in 1% Sarkosyl NL97 for 15 min at 60 °C, and then digested by Pronase (final concentration 5 mg/ml) for 2 h at 37 °C. A 0.5 ml aliquot of the sample was mixed with 9-5 ml of CsCl (initial density 1.700 g/ml), prepared in TES buffer, and centrifuged at 28000 r.p.m. for 64 h at 18 °C in the 50Ti rotor. Fractions were collected from the top of the gradient by means of a density gradient fractionator (Gilson), and radioactivity incorporated into 10% trichloroacetic acid-insoluble material was counted in a Beckman scintillation counter.

*Polyacrylamide gel electrophoresis (PAGE).* HEL cells grown in 60 mm Petri dishes were infected with HCMV at an m.o.i. of 1 or 5, or mock-infected. After 1 h adsorption, the cells were washed twice with HBSS and then labelled with 30 μCi [3S]methionine (sp. act. 110 Ci/mmol, Amersham) per ml for 2 h in methionine-free maintenance medium. The cultures were washed three times with ice-cold phosphate-buffered saline. Immediate early polypeptides were extracted, precleared of extraneous protein by incubation with HCMV-negative human serum and then immunoprecipitated by immediate early antigen (IEA)-positive human serum according to a method described previously (Michelson et al., 1979; Blanton & Tevethia, 1981). Immunoprecipitated polypeptides were separated by electrophoresis on 10% slab gels (Laemmli, 1970). After electrophoresis, gels were fixed, soaked in En3Hance (New England Nuclear) and dried. The autoradiogram was recorded on Kodak X-Omat film. [35S]Methionine-labelled Sendai virus structural polypeptides were co-electrophoresed on each gel as molecular weight standards.

**RESULTS**

*Effect of dexamethasone on plaquing efficiency and production of HCMV.*

To study the effect of adrenal corticosteroids on the multiplication of HCMV, we first determined plaquing efficiency and production of HCMV in HEL cells treated with various concentrations of dexamethasone. A synthetic glucocorticoid. As shown in Table 1, plaque numbers found of HEL cells treated with 10^-5 to 10^-8 m-dexamethasone were five- to 11-fold higher than those on untreated control cultures. In addition, individual plaques appeared earlier and were larger on the hormone-treated HEL cells as compared with untreated cultures; plaques first appeared after 4 days on 10^-5 m-dexamethasone-treated HEL cells in contrast to the first appearance at 7 days post-infection on control cultures; plaque size on the hormone-treated cultures was 1-5- to 2-fold larger than that on the control when measured at 14 days. The yield of HCMV in the hormone-treated HEL cells was also enhanced 2-5- to 14-3-fold over production in untreated cultures (Table 1).
HCMV replication in hormone-treated cells

Fig. 1. Growth kinetics of HCMV in untreated or dexamethasone-treated HEL cells. The cells either untreated or pretreated for 24 h with the hormone (10^{-5} M) were infected with HCMV at an m.o.i. of 1. After 90 min adsorption the cells were washed with HBSS and maintenance medium with or without the hormone was added to the respective cells. At the indicated times after virus infection, the total amount of infectious virus was determined by plaque assay. Medium was changed at 3 days. O, Untreated control cultures; ●, dexamethasone-treated cultures.

Table 1. Effect of various concentrations of dexamethasone on plaquing efficiency and production of HCMV

<table>
<thead>
<tr>
<th>Dexamethasone (M)</th>
<th>No. of plaques per plate*</th>
<th>Virus yield† (p.f.u./ml x 10^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6</td>
<td>2.2</td>
</tr>
<tr>
<td>10^{-8}</td>
<td>30</td>
<td>5.5</td>
</tr>
<tr>
<td>10^{-7}</td>
<td>42</td>
<td>12.5</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>51</td>
<td>30.5</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>66</td>
<td>31.5</td>
</tr>
</tbody>
</table>

* Confluent monolayers of HEL cells, grown in 60 mm Petri dishes, were pretreated for 24 h with the indicated concentration of dexamethasone. The cells were infected with HCMV at an m.o.i. of approx. 10 p.f.u./plate. After 90 min adsorption the plates were overlaid with maintenance medium containing 0.4% agarose and the indicated concentration of the hormone. Seven days later a second overlay was added to the plates. The plaques were scored at 14 days; average plaque numbers in each of two samples are shown.

† HEL cells pretreated for 24 h with the indicated concentration of dexamethasone were infected with HCMV at an m.o.i. of 1. After 90 min adsorption, maintenance medium containing the indicated concentration of the hormone was added. At 72 h post-infection the total amount of infectious virus was determined.

Growth kinetics of HCMV in untreated or dexamethasone-treated HEL cells

Growth of HCMV in either untreated or the hormone-treated cultures was assessed by infecting monolayers at an m.o.i. of 1. A typical experiment is shown in Fig. 1. The synthesis of infectious HCMV was first observed at 3 days in untreated control cultures. In contrast, in the hormone-treated cultures infectious progeny virus appeared 2 days after infection and the amount of infectious virus produced during 3 to 6 days post-infection was consistently about 10-fold higher than that in the control.
Table 2. Effect of various steroid hormones on replication of HCMV in HEL cells*

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Hormone†</th>
<th>Virus yield‡ (p.f.u./ml × 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>Dexamethasone</td>
<td>127.5</td>
</tr>
<tr>
<td></td>
<td>Hydrocortisone</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>Prednisolone</td>
<td>22.5</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>β-Oestradiol</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>Dexamethasone + progesterone</td>
<td>62.5</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>Hydrocortisone</td>
<td>51.7</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Hydrocortisone + progesterone</td>
<td>24.5</td>
</tr>
</tbody>
</table>

* HEL cells either untreated or pretreated for 24 h with the indicated steroid hormone were infected with HCMV at an m.o.i. of 0.1. After 90 min adsorption, maintenance medium containing the indicated hormone was added.
† The concentration of each hormone was 10⁻⁶ M.
‡ Titrated 72 h after virus infection.

Effect of other steroid hormones on production of HCMV

To determine whether this enhancing activity by dexamethasone is due to glucocorticoid activity, experiments using other steroid hormones were carried out (Table 2). Dexamethasone enhanced the production of HCMV 34.4-fold over that in untreated cultures. Two natural glucocorticoid hormones enhanced the production of HCMV by levels ranging from 6.0-fold (prednisolone) to 6.8-fold (hydrocortisone). However, oestrogenic (progesterone and β-oestradiol) and androgenic (testosterone) hormones did not enhance HCMV multiplication. Because progesterone has been shown to act as a glucocorticoid hormone antagonist by competing for cytoplasmic receptor protein (Samuels & Tomkins, 1970), we tested the effect of progesterone on the enhancement of HCMV production by dexamethasone or hydrocortisone. The enhancing activity by dexamethasone or hydrocortisone was reduced by 50% when infected cells were treated simultaneously with these hormones and progesterone (Table 2). These results indicate that the mechanism resulting in enhanced production of HCMV is specific for adrenal glucocorticoid hormones and suggest that the hormonal enhancement of HCMV production involves specific receptor proteins in target cells.

Virus adsorption and cell growth

A possible mechanism involved in the enhanced production of HCMV by dexamethasone is hormone enhancement of virus adsorption or stimulation of cell growth. It is known that HCMV replication in growing cells is faster and greater than that in resting cells (DeMarchi & Kaplan, 1977). To test this possibility, the rate of cell growth and virus adsorption were examined. No significant difference was observed in the cell growth in either untreated or hormone-treated cells. To determine the adsorption rate of HCMV, monolayers of HEL cells, either untreated or pretreated with dexamethasone, were exposed to partially purified HCMV that had been labelled with [³H]thymidine. The cultures were incubated at 37 °C. At 30 min intervals the inoculum was removed from each of two samples and residual acid-insoluble radioactivity in the supernatant was counted. Pretreatment of HEL cells with the hormone did not affect the rate of virus adsorption during the 90 min adsorption period (data not shown).

Analysis of HCMV DNA synthesis in dexamethasone-treated cells

The rate of HCMV DNA synthesis in dexamethasone-treated HEL cells was compared to that in untreated control cultures. HEL cells untreated or pretreated with dexamethasone were
HCMV replication in hormone-treated cells

Fig. 2. Analysis of DNA isolated from untreated or dexamethasone-treated HEL cells infected with HCMV. HEL cells either untreated or pretreated for 24 h with the hormone (10^{-5} M) were infected with HCMV at an m.o.i. of 1. The cells were pulse-labelled with [3H]thymidine from 24 to 48 h after infection in the presence or absence of the hormone. DNA was extracted and virus DNA was separated from cellular DNA by isopycnic centrifugation in CsCl as described in Methods. The amount of [3H]-thymidine incorporated into acid-insoluble material was determined. (a) DNA extracted from untreated control cultures; (b) DNA extracted from dexamethasone-treated cultures.

Table 3. Production of HCMV in HEL cells treated with dexamethasone at various times before and after virus infection*

<table>
<thead>
<tr>
<th>Period of dexamethasone treatment (h)†</th>
<th>Virus yield‡ (p.f.u./ml \times 10^{-5})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>3.0</td>
</tr>
<tr>
<td>-24-0</td>
<td>13.2</td>
</tr>
<tr>
<td>1.5-96</td>
<td>28.5</td>
</tr>
<tr>
<td>24-96</td>
<td>18.7</td>
</tr>
<tr>
<td>48-96</td>
<td>7.7</td>
</tr>
<tr>
<td>72-96</td>
<td>5.5</td>
</tr>
<tr>
<td>-24.0, 1.5-96</td>
<td>37.0</td>
</tr>
</tbody>
</table>

* HEL cells were treated with dexamethasone (10^{-5} M) for the indicated period before and after virus infection at an m.o.i. of 1.
† The time of virus infection is taken as 0 time.
‡ Titrated at 96 h post-infection.

infected with HCMV at an m.o.i. of 1, and pulsed with [3H]thymidine from 24 to 48 h post-infection in the presence or absence of the hormone. Virus DNA and host cell DNA in cellular extracts were separated by equilibrium centrifugation in CsCl as described in Methods. Fig. 2 illustrates the sedimentation profiles. In the hormone-treated cultures (Fig. 2b) the peak (density 1.717 g/ml) that corresponds to the density of standard HCMV DNA (Plummer et al., 1969; St. Jeor & Rapp, 1973) was about three times larger than that in control cultures (Fig. 2a). However, the peak at the density of mammalian cell DNA (Crouch & Rapp, 1972) was not significantly different between the two cases. These results indicate that the synthesis of virus DNA is enhanced in the dexamethasone-treated HEL cells.

To determine the stages in the HCMV replication cycle that are under hormonal control, HEL cell monolayers were treated with dexamethasone at various times before and after virus infection and virus yield was determined at 96 h (Table 3). The yield of virus was significantly enhanced in cultures treated with the hormone prior to virus infection (- 24 to 0 h) or during the 48 h latent period of virus replication (1.5 to 96 h and 24 to 96 h). However, a little enhancement
Fig. 3. Analysis of immediate early proteins synthesized in untreated or dexamethasone-treated HEL cells by SDS-PAGE. HEL cells were either untreated or pretreated for 24 h with the hormone (10^-5 M). These cells were infected with HCMV at an m.o.i. of 1 (e, f) or 5 (c, d), or mock-infected (a, b). After a 1 h adsorption period the cells were washed and pulse-labelled with [3S]methionine for 2 h in methionine-free maintenance medium with or without the hormone. (b, d, f) Untreated control cultures; (a, c, e) dexamethasone-treated cultures.

was observed in cultures treated with the hormone after virus synthesis began (48 to 96 h and 72 to 96 h).

Effect of dexamethasone on the synthesis of immediate early proteins and antigens

During the course of these experiments, we noticed that in the hormone-treated HEL cells early c.p.e., resulting from the synthesis of early proteins (Michelson-Fiske et al., 1977; Stinski, 1978), appeared earlier and in more cells than in untreated control cultures. Therefore, we examined the synthesis of immediate early proteins in the hormone-treated cells by SDS-PAGE analysis. The results, illustrated in Fig. 3, show that IEA-positive human serum mainly precipitated a band with an apparent molecular weight of 72000 in all virus-infected cells (lanes c to f), but not in either untreated or dexamethasone-treated mock-infected cells (lanes a and b). The relative amount of synthesis of the protein was enhanced in the hormone-treated cultures infected with HCMV at an m.o.i. of 5 (lane c) or 1 (lane e) compared to the respective control cultures (lanes d and f). Moreover, in the hormone-treated cultures infected with virus at an
HCMV replication in hormone-treated cells

Table 4. Effect of cordycepin on enhancing activity by dexamethasone*

<table>
<thead>
<tr>
<th>Dexamethasone (Μ)</th>
<th>Cordycepin (μg/ml)</th>
<th>Virus yield† (p.f.u./ml × 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1·2</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>2·2</td>
</tr>
<tr>
<td>0</td>
<td>30</td>
<td>1·4</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>0</td>
<td>8·7</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>20</td>
<td>3·5</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>30</td>
<td>1·8</td>
</tr>
</tbody>
</table>

* HEL cells were pretreated with the indicated concentration of dexamethasone, cordycepin or both for 24 h before virus infection at an m.o.i. of 0·1.
† Titrated at 72 h post-infection.

m.o.i. of 5, another polypeptide (apparent mol. wt. 76000) was also found in addition to a 72000 polypeptide (lane c).

The synthesis of IEA was also stimulated in the hormone-treated cells. HEL cells infected with virus at an m.o.i. of 0·1 were fixed with methanol at 3 h and stained by standard indirect immunofluorescence techniques (Tanaka et al., 1981) using the same serum as used in the immunoprecipitation. In untreated control cultures, IEA was found in 1 to 3% of the cells. In contrast, in the hormone-treated cells 8 to 10% of the cells contained IEA.

Effect of cordycepin on the enhancement of HCMV production by dexamethasone

To determine whether the enhancement of HCMV production by dexamethasone is dependent on the synthesis of a cellular mRNA(s) as a result of hormonal action, like hormonal enzyme induction (Scott et al., 1972), the effect of cordycepin on the enhanced production of HCMV in the hormone-treated cultures was studied. This experiment is based on the fact that cordycepin inhibits the maturation of mRNA by blocking poly(A) synthesis (Penman et al., 1970; Darnell et al., 1971; Abelson & Penman, 1972). The results are shown in Table 4. When cells were pretreated with dexamethasone, the yield of HCMV was enhanced by approximately sevenfold. Pretreatment with cordycepin alone did not affect the virus yield. In the cultures pretreated simultaneously with the hormone and cordycepin at concentrations of 20 or 30 μg/ml, there was about 60 or 80% reduction in virus yield when compared to the yield in the cells treated with dexamethasone alone.

DISCUSSION

The data presented in this paper indicate that treatment of cells with pharmacological concentrations of dexamethasone enhances HCMV replication in HEL cells. This enhanced replication of HCMV is demonstrated by several biological and biochemical parameters, i.e. enhanced plaquing efficiency, earlier appearance of c.p.e., shortened virus eclipse period, increased synthesis of immediate early proteins and antigens, and enhanced virus DNA synthesis.

In these studies we have found that the hormonal enhancement of HCMV replication probably involves specific receptor proteins in target cells and requires the synthesis of a cellular mRNA(s). Because androgenic and oestrogenic hormones do not significantly enhance HCMV replication, this involvement of transcription in enhancement may be specifically induced by glucogenic hormones. This may be because androgenic and oestrogenic hormones have different modes of action or because fibroblast cells are not targets for these two hormones.

In our system, the early stages in HCMV replication cycle seem to be under hormonal control. It is likely that the enhancing effect of the hormone is expressed through host cellular functions. Evidence that this may be the case comes from the fact that when cultures are pretreated with the hormone, which is then removed before infection, the yield of progeny virus is significantly higher than that in untreated control cultures (Tables 3 and 4). However, the possibility that dexamethasone may stimulate HCMV transcription directly cannot be ruled out, because the
yield of HCMV is further enhanced when the hormone is allowed to remain in the cultures throughout the experiment (Table 3). This problem is now under investigation.

The same hormone has been found to enhance production of murine leukaemia virus (Paran et al., 1973), murine mammary tumour virus (Parks & Scolnick, 1974), polyoma virus (Morhenn et al., 1973) and a certain strain of HSV type 2 (Costa et al., 1974). However, whether these phenomena have similar molecular mechanisms is unknown.

Under natural conditions, HCMV causes many diseases after primary or reactivation infection. Chiba et al. (1972) have suggested that corticosteroid hormones are one risk factor for HCMV infection in children. Another cytomegalovirus, murine cytomegalovirus, has been shown to be reactivated from latent virus infection by treatment of the animals with cortisone and anti-lymphocyte serum (Jordan et al., 1977). Moreover, recently we have found that dexamethasone also enhances HCMV replication in human epithelial cells in which HCMV replication occurs most frequently in vitro (Tanaka et al., 1984). These observations, coupled with the findings described here, suggest that glucocorticoid steroid hormones could play a biologically significant role in HCMV-cell interactions in vitro.

REFERENCES


(Received 9 January 1984)
We present the results of a multicenter clinical trial using Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes (CTLs) generated from EBV-seropositive blood donors to treat patients with EBV-positive posttransplantation lymphoproliferative disease (PTLD) on the basis of the best HLA match and specific in vitro cytotoxicity. Thirty-three PTLD patients who had failed on conventional therapy were enrolled. No adverse effects of CTL infusions were observed and the response rate (complete or partial) in 33 patients was 64% at 5 weeks and 52% at 6 months. Fourteen patients achieved a complete remission, 3 showed a partial response, and 16 had no response at 6 months (5 died before completing treatment). At 5 weeks, there was a significant trend toward better responses with higher numbers of CD4+ cells in infused CTL lines (P = .001) that were maintained at 6 months (P = .001). Patients receiving CTLs with closer HLA matching responded better at 6 months (P = .048). Female patients responded better than male patients, but the differences were not statistically significant. Our results show that allogeneic CTLs are a safe and rapid therapy for PTLD, bypassing the need to grow CTLs for individual patients. The response rate in this poor prognosis patient group is encouraging. (Blood. 2007;110:1123-1131) © 2007 by The American Society of Hematology

Introduction

Posttransplantation lymphoproliferative disease (PTLD) is a common complication of solid organ transplantation that carries a high morbidity and mortality.1 The majority of tumors are of B-cell origin and are associated with Epstein-Barr virus (EBV). EBV is a herpes virus that induces proliferation and transformation of B lymphocytes in vitro and is associated with several human B-cell tumors including Burkitt lymphoma and Hodgkin disease.2,3 Most people acquire EBV silently during early childhood, but if primary infection is delayed until after puberty then it is associated with infectious mononucleosis (IM) in around 25% of cases.4 More than 90% of adults carry EBV as a persistent infection of B lymphocytes. Cytotoxic T lymphocytes (CTLs) that recognize both latent and lytic viral antigens are important in controlling EBV reactivation.5 EBV-specific CTLs are readily detectable in peripheral blood of healthy seropositive carriers, but in transplant recipients, who require immunosuppressive drugs, the activity of virus-specific CTLs is suppressed, thus predisposing the patients to PTLD. In addition primary EBV infection following transplantation carries a high risk of PTLD, and since most recipients who are EBV seronegative before transplantation are children, there is an increased incidence of the PTLD in the pediatric transplant population.6,7

PTLD is most common within the first year following transplantation during high-dose immunosuppression, but may occur at any stage, even after several decades.8 The clinical presentation of PTLD is variable and it is often difficult to diagnose. Lesions may be single or multiple, nodal or extra nodal—common sites being the gut, brain and transplanted organ. PTLD may present as an IM-like syndrome particularly following primary EBV infection. The tumor encompasses a spectrum of histologic types ranging from hyperplasia to monomorphic lymphoma.9 Early lesions may be polyclonal with progression to monoclonality. In EBV-associated PTLD, tumor cells contain viral DNA and express the small EB-encoded RNAs (EBERS) and a variety of viral proteins. Most tumors show full latent viral gene expression (EB nuclear antigens [EBNAs] 1, 2, 3a, 3b, 3c, and leader protein [LP]; latent membrane proteins [LMPs] 1, 2), with a few cells expressing lytic cycle genes. Expression of the EBNAs 3 proteins, which are immunodominant for CTL responses, indicates that most PTLD tumors grow opportunistically in the absence of effective T-cell control of the persistent virus infection. However viral antigen expression is variable within and between tumors, some showing more restricted forms of latency either without evidence of EBNAs 2 and 3 proteins, or a Burkitt-like EBNAs 1 only phenotype.9

First-line treatment for PTLD is immunosuppression dose reduction, which is successful in inducing tumor regression in a proportion of cases.10 However this treatment may be limited by the onset of graft rejection due to reduced immunosuppression, and
other forms of treatment such as chemotherapy, radiotherapy, and rituximab (anti-CD20 monoclonal antibody) are often required.\textsuperscript{11,12} Despite these treatments, the overall mortality from PTLD in solid organ transplantation is around 50%.\textsuperscript{13} Another approach to PTLD treatment is to enhance the EBV-specific CTL response that is suppressed in transplant recipients. Cytotoxic T-cell therapy to prevent and/or treat PTLD was first used successfully in allogeneic hematopoietic stem cell transplant recipients where stem cell donor’s peripheral blood was used as a source of EBV-specific CTLs for infusion.\textsuperscript{14-16} However, since donor blood is not generally available for solid organ recipients with PTLD, we established a frozen bank of 100 EBV-specific CTLs generated from the peripheral blood of Scottish blood donors.\textsuperscript{17} We previously conducted a pilot study in which 8 patients with progressive PTLD were treated with these CTLs selected on the basis of best HLA matches between the CTL donor and PTLD patient. Three patients attained complete remission.\textsuperscript{18} In this present study, we used these partially HLA-matched allogeneic CTLs in a phase 2 multicenter clinical trial to treat 33 patients (31 solid organ and 2 stem cell transplant recipients) with biopsy proven EBV-positive PTLD that had failed conventional treatment. The overall response rate was 64% at 5 weeks and 52% at 6 months.

**Patients, materials, and methods**

**Study design**

This phase 2 multicenter clinical trial aimed to test the safety and efficacy of banked allogeneic EBV-specific CTLs used on a best HLA-match basis to treat PTLD. Inclusion criteria were as follows: biopsy-proven EBV-positive PTLD and a tumor where response could be measurable. Exclusion criteria were as follows: pregnancy and Karnofsky scale of 10 or lower. Standard therapy involved weekly intravenous infusions of CTLs ($2 \times 10^6$ CTLs per kg body weight) for 4 weeks—doses established previously in a pilot study.\textsuperscript{19} Patients from 19 transplantation centers were recruited to the trial (listed in “Acknowledgments”) with informed written consent from patients or guardians obtained in accordance with the Declaration of Helsinki. Preliminary data from the first 7 patients included in this trial were published elsewhere.\textsuperscript{18} The trial was approved by the Multicentre Research Ethics Committee for Scotland and by local research ethics committees for each center. This trial was also registered with European Clinical Trial Database, ClinicalTrials.gov, and the United Kingdom National Cancer Research Network.

**Cytotoxic T-cell bank and in vitro testing**

The establishment and management of the CTL bank have been reported elsewhere.\textsuperscript{17} Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from HLA-typed, EBV-seropositive blood donors at Scottish National Blood Transfusion Services with informed consent. Lymphoblastoid cell lines (LCLs) were established by in vitro EBV infection and used as stimulators to grow EBV-specific CTLs by published methods.\textsuperscript{17} CTLs were tested in standard chromium release assays for cytotoxicity against autologous phytohemagglutinin (PHA)-stimulated blasts, autologous and mismatched LCLs, and K562 cell line to detect NK-cell activity. When deemed EBV specific, CTLs were screened for bacterial, viral, and fungal contaminants; stained and analyzed by fluorescence-activated cell sorting (FACS) (for TCRβ, CD3, CD4, CD8, CD19, CD16/7, CD56, CD57, and a wide range of T-cell activation and differentiation markers); and then frozen in vials until required.\textsuperscript{17}

**Patient/CTL matching procedure**

HLA A and B antigens were typed by a one-stage microcytotoxicity test using the First and Second Lambda monoclonal class I 72-well trays (One Lambda, VH Bio, Newcastle, United Kingdom) and HLA DR by low-resolution polymerase chain reaction (PCR).\textsuperscript{17} The HLA profile (A, B, DR) of the potential CTL recipients was used to identify the closest matched banked CTLs and 2 to 3 CTLs were thawed and tested for cytotoxicity in chromium release assays against autologous LCLs and PHA blasts, K562, patient PHA blasts, and, where available, patient LCLs. The CTLs showing the highest specific killing against patient LCLs with low killing of patient PHA blasts and K562 were selected for infusion.\textsuperscript{19}

**Immunohistochemistry**

The diagnosis of PTLD and its classification were determined by routine histologic examination. EBER staining was carried out by in situ hybridization using a commercial kit (Dako, Cambridge, United Kingdom), and EBNA 1 and 2, and LMP 1 expression were detected by routine staining using commercially available antibodies (EBNA 2 no. M7004, LMP-1 no. M0897 from Dako; EBNA 1 no. Ab8329 from AbCam, Cambridge, United Kingdom) and detection system (Dako EnVision kit) after antigen retrieval (pressure cooking 3 minutes at pressure in Tris/EDTA). Tumor cell cloneology was assessed by in situ hybridization for κ and λ mRNA (Dako).

**Patient monitoring**

Tumor response (complete, partial, or no response) was recorded 5 weeks and 6 months after CTL therapy as determined by clinical evaluation, imaging (x-ray, computed tomography [CT], magnetic resonance imaging [MRI] scan as appropriate) or endoscopy, and was decided by the physicians involved in patient care. Clinical monitoring was carried out weekly for 4 weeks and then at regular intervals for 6 months. A complete response (CR) was defined as complete disappearance of all measurable tumor masses and any other manifestations of disease.\textsuperscript{19} A partial response (PR) was achieved if the overall tumor mass decreased 50% or more in size where tumors were measurable clinically and/or by imaging and the patient’s clinical condition remained stable or improved. No response (NR) was defined as tumor masses that remained the same size or increased in size and/or the patient’s clinical condition deteriorated.

**Quantitative EBV load by real-time PCR**

EBV DNA levels were monitored by real-time PCR of EBV polymerase gene using Corbett Rotor-gene 3000 machine (Corbett Research, Cambridge, United Kingdom) in PBMCs taken before and after each CTL infusion and thereafter at clinical monitoring sessions.\textsuperscript{20} Briefly, DNA was extracted using QIAamp DNA Mini Kit as per the manufacturer’s instructions (Qiagen, Crawley, United Kingdom) and stored at $-70^\circ$C. DNA (5 microliters [μl]/1 μg) was then amplified in a 25-μl reaction volume containing primers and Taq polymerase (Promega, Southampton, United Kingdom). EBV-positive Raji cells were used to obtain a standard curve from a dilution series starting with 100 μg DNA/tube and then 10-fold serial dilutions to give a final concentration of 10 pg DNA/tube. Viral DNA copy number in test samples was estimated by extrapolating from the Raji standard curve. β-Globin was always included in each PCR run as a house keeping gene. EBV load in test samples was expressed as the relative copy number normalized against the amount of amplified β-globin DNA.

**T-cell receptor (TCR) spectratyping**

Functionally rearranged TCR β chain variable (BV) gene subfamilies were amplified across the complementarity determining region 3 (CDR3) encoding regions using 23 subfamily-specific primers and a FAM-conjugated β chain constant region–specific primer.\textsuperscript{21,22} DNA was extracted from 5 × 10^6 PBMCs or CTLs using the Rneasy mini kit and Qiagen kit (Qiagen) as per the manufacturer’s instructions and was converted to complementary DNA (cDNA) using random hexamers and the ThermoScript RT system from Invitrogen (Paisley, United Kingdom). PCR amplifications were performed on 1 μl cDNA in a total volume of 20 μl containing variable and constant primers and 0.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA) polymerase. PCR product (1 μl) was diluted in 10 μl nuclease-free water and then further diluted 1 in 10 with Hi-Di formamide (containing Genescan 500LIZ size standard) before
electrophoresis in an ABI 3730 (Dye set 5) automated sequencer. ABI Genemapper software (version 3.7) was used to analyze data (Applied Biosystems).

**Allotransplantation and PTLD development and level of EBV-specific CTL killing)**

Patient 21 had a further course of reduction of immunosuppression (patient 21, she was in CR at the time of death), and one patient who showed a partial response to CTL therapy at 5 weeks died 3 months later from severe cytomegalovirus infection (patient 22). Two patients with partial responses at 5 weeks achieved complete response before completion of CTL treatment were classified as nonresponders.

The overall survival rate is shown in Figure 1. At 6 months, 26 patients (79% survival) were still alive (Tables 1, 2). One patient who showed a partial response to CTL therapy at 5 weeks died 3 months later from severe cytomegalovirus infection (patient 21, she was in CR at the time of death), and one patient from the NR group died due to an extensive tumor burden (patient 22). Three patients who initially had shown a partial response relapsed by 6 months.

Two patients with partial responses at 5 weeks achieved complete response at 6 months bringing the number of patients showing CR to CTL therapy to 14. One CR patient has since relapsed and 13 remain disease free, 1 to 7.5 years after completing CTL therapy.

Two of the 3 patients (patients 15 and 16) with PR at 6 months are still alive (18 and 20 months after infusions, respectively). Patient 15 had a further course of reduction of immunosuppression. The third PR patient (patient 17) had a relapse and died 7 months later.

Thus, overall a response (complete or partial) to CTL therapy was recorded in 21 (64%) of 33 patients at 5 weeks and 17 (52%) of 33 patients at 6 months. Because the number of participants is relatively small, partial and complete responders were aggregated for comparison with the nonresponder group in further analyses.

**Response in female compared with male patients**

The response was better among female than male patients at both 5 weeks (79% versus 53%, respectively) and 6 months (71% versus 37%, respectively), but these differences were not statistically significant (Table 2). However, when analyses were restricted to 28 patients who successfully completed treatment, the results became significant: at both 5 weeks and 6 months all (11 of 11 and 10 of 10, respectively) female patients showed a response, whereas at the same time points, 10 of 17 and 7 of 16 male patients responded ($P = .02$ at 5 weeks and $P = .004$ at 6 months). There was no association between the sex of CTL donors or CD4 T-cell counts in CTLs and sex-related response (data not shown). The age of the patients did not affect outcome.
EBV serostatus, time of onset of PTLD, and outcome

Pretransplantation EBV serology was available from 17 of 33 patients (Table 1). Ten patients were seronegative before transplantation and developed PTLD following primary EBV infection. Seven (70%) of these 10 previously EBV-seronegative patients showed a response (6 CR and 1 PR) to CTL at 6 months compared with 2 (29%) of 7 patients who were already EBV seropositive before transplantation.

The interval between transplantation and PTLD development varied from 6 weeks to 19 years (median time, 3.5 years). The response at 5 weeks to CTL therapy did not differ significantly between early and late onset of disease (Table 2): 9 (64%) of 14 early (PTLD < 2 years after transplantation) compared with 12 (63%) of 19 late (PTLD > 2 years after transplantation) PTLD responded ($P = .999$). At 6 months, all 9 of 14 patients with early onset disease showed a sustained response, whereas only 42% (8/19) of patients with late onset PTLD maintained the initial response ($P = .30$).

### Degree of HLA matching

CTL lines used in the trial were selected for individual patients on the basis of the number of HLA matches (A, B, and DR), with preference given to HLA class I antigen (A and B) matching as EBV antigens expressed in relation to HLA class I molecules are known to be the main targets for CTLs. The number of HLA matches at HLA A, B, and DR loci varied from 2 of 6 to 5 of 6, and

### Table 1. Patients’ characteristics and outcome of CTL infusions

<table>
<thead>
<tr>
<th>Pt no.</th>
<th>Sex</th>
<th>Tx/time to PTLD</th>
<th>Age at CTL, y</th>
<th>Pre-Tx EBV*</th>
<th>PTLD sites</th>
<th>PTLD histology†</th>
<th>Pre-CTL EBV DNA‡</th>
<th>Previous Rx for PTLD</th>
<th>Outcome, 5 wk</th>
<th>Outcome, 6 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 F</td>
<td>Liver/3 mo</td>
<td>1</td>
<td>Pos</td>
<td>Tonsil, stomach, bowel</td>
<td>Monomorphic</td>
<td>10$^6$</td>
<td>RIS, ACV</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>2 F</td>
<td>Liver/6.5 y</td>
<td>76</td>
<td>NK</td>
<td>Bowel</td>
<td>Hodgkin</td>
<td>ND</td>
<td>RIS, surgery</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>3 M</td>
<td>Heart/5.5 y</td>
<td>51</td>
<td>NK</td>
<td>Orbit, pectoral</td>
<td>Monomorphic</td>
<td>454</td>
<td>RIS</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>4 F</td>
<td>Stem cell/1.5 mo</td>
<td>30</td>
<td>Pos</td>
<td>LN (multisites), blood</td>
<td>Polymorphic</td>
<td>ND</td>
<td>RIS, rituximab</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>5 M</td>
<td>Bone marrow/4 mo</td>
<td>27</td>
<td>NK</td>
<td>LN</td>
<td>Polymorphic</td>
<td>ND</td>
<td>RIS, Chemo, radio</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>6 F</td>
<td>Kidney/9 y</td>
<td>68</td>
<td>NK</td>
<td>LN, bone marrow</td>
<td>Burkitt</td>
<td>24 000</td>
<td>RIS, chemo</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>7 F</td>
<td>Kidney/19 y</td>
<td>67</td>
<td>NK</td>
<td>LN (multisites)</td>
<td>Hodgkin</td>
<td>ND</td>
<td>RIS</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>8 F</td>
<td>Liver/9 mo</td>
<td>13</td>
<td>Neg</td>
<td>Bowel, LN</td>
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<td>ND</td>
<td>RIS, VCV</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>9 F</td>
<td>Liver/3 mo</td>
<td>50</td>
<td>NK</td>
<td>LN (multisites)</td>
<td>Hodgkin</td>
<td>ND</td>
<td>RIS, radio</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>10 M</td>
<td>Liver, small bowel/21 mo</td>
<td>3</td>
<td>Neg</td>
<td>Sigmoid colon, duodenum</td>
<td>Hyperplastic</td>
<td>1200</td>
<td>RIS</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>11 F</td>
<td>Liver/4.2 y</td>
<td>5</td>
<td>Neg</td>
<td>LN</td>
<td>Hyperplastic</td>
<td>339</td>
<td>RIS</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>12 M</td>
<td>Liver, small bowel/9 mo</td>
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<td>Neg</td>
<td>Small bowel, LN</td>
<td>Hodgkin</td>
<td>1052</td>
<td>RIS, GCV</td>
<td>CR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>13 F</td>
<td>Kidney/23 mo</td>
<td>19</td>
<td>Neg</td>
<td>Brain (multiple)</td>
<td>Polymorphic</td>
<td>ND</td>
<td>RIS, rituximab, chemo, radio</td>
<td>PR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td>14 F</td>
<td>Kidney/23 mo</td>
<td>35</td>
<td>Neg</td>
<td>Brain (multiple)</td>
<td>Monomorphic</td>
<td>ND</td>
<td>RIS</td>
<td>PR</td>
<td>CR</td>
<td></td>
</tr>
<tr>
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<td>Kidney/3.5 y</td>
<td>41</td>
<td>Neg</td>
<td>Gingivae, adrenals</td>
<td>Monomorphic</td>
<td>4000</td>
<td>RIS</td>
<td>PR</td>
<td>PR</td>
<td></td>
</tr>
<tr>
<td>16 M</td>
<td>Kidney/18.5 y</td>
<td>64</td>
<td>NK</td>
<td>Kidney, bladder, LN</td>
<td>Monomorphic</td>
<td>100 000</td>
<td>RIS, chemo, radio</td>
<td>PR</td>
<td>PR</td>
<td></td>
</tr>
<tr>
<td>17 M</td>
<td>Liver/4.3 y</td>
<td>60</td>
<td>NK</td>
<td>Liver, abdomen LN</td>
<td>Polymorphic</td>
<td>640</td>
<td>RIS</td>
<td>PR</td>
<td>PR</td>
<td></td>
</tr>
<tr>
<td>18 M</td>
<td>Kidney/5.3 y</td>
<td>11</td>
<td>Pos</td>
<td>Tonsil, LN (multisites)</td>
<td>Polymorphic</td>
<td>1.2 × 10$^6$</td>
<td>RIS</td>
<td>PR</td>
<td>NR</td>
<td></td>
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<tr>
<td>19 M</td>
<td>Heart/20 y</td>
<td>33</td>
<td>NK</td>
<td>LN (multisites)</td>
<td>Hyperplastic</td>
<td>ND</td>
<td>RIS</td>
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<td>NR</td>
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<tr>
<td>20 M</td>
<td>Liver/9 y</td>
<td>14</td>
<td>Neg</td>
<td>LN (multisites)</td>
<td>Hodgkin</td>
<td>ND</td>
<td>RIS</td>
<td>PR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>21 F</td>
<td>Heart, lungs/3 y</td>
<td>51</td>
<td>Pos</td>
<td>Brain (multiple)</td>
<td>Monomorphic</td>
<td>ND</td>
<td>RIS, rituximab, chemo, radio</td>
<td>PR</td>
<td>NR; died</td>
<td></td>
</tr>
<tr>
<td>22 M</td>
<td>Kidney/8 y</td>
<td>61</td>
<td>Pos</td>
<td>Large pelvic mass (kidney, bladder)</td>
<td>Monomorphic</td>
<td>24 000</td>
<td>RIS, rituximab, chemo, radio</td>
<td>NR</td>
<td>NR; died</td>
<td></td>
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<tr>
<td>23 M</td>
<td>Liver/4.7 y</td>
<td>49</td>
<td>NK</td>
<td>Blood, bone marrow</td>
<td>Polymorphic</td>
<td>ND</td>
<td>RIS</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>24 M</td>
<td>Kidney/10 y</td>
<td>48</td>
<td>NK</td>
<td>Eye, spine, bladder, LN</td>
<td>Monomorphic</td>
<td>7900</td>
<td>RIS, rituximab</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
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<td>Kidney/8.8 y</td>
<td>51</td>
<td>NK</td>
<td>LN (multisites)</td>
<td>Monomorphic</td>
<td>50 752</td>
<td>RIS</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>26 M</td>
<td>Kidney/13 y</td>
<td>19</td>
<td>NK</td>
<td>Brain (multiple)</td>
<td>Polymorphic</td>
<td>3200</td>
<td>RIS</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>27 M</td>
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<td>NK</td>
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<td>704</td>
<td>RIS, rituximab, Anti-IL6, GCV</td>
<td>NR</td>
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<td>61</td>
<td>NK</td>
<td>LN (multisites)</td>
<td>Monomorphic</td>
<td>1827</td>
<td>RIS</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>29 M</td>
<td>Kidney/6 y</td>
<td>53</td>
<td>NK</td>
<td>Lungs, LN</td>
<td>Polymorphic</td>
<td>ND</td>
<td>RIS, rituximab, chemo</td>
<td>NR; died d8</td>
<td>Nf; died</td>
<td></td>
</tr>
<tr>
<td>30 F</td>
<td>Lung/3 mo</td>
<td>58</td>
<td>Pos</td>
<td>Lung, LN</td>
<td>Monomorphic</td>
<td>ND</td>
<td>RIS, rituximab, chemo NR–Died d11</td>
<td>Nf; died</td>
<td>Nf; died</td>
<td></td>
</tr>
<tr>
<td>31 F</td>
<td>Kidney/6 mo</td>
<td>12</td>
<td>Neg</td>
<td>Kidney, spleen, liver, bone marrow, pleura</td>
<td>Hyperplastic</td>
<td>2127</td>
<td>RIS, rituximab</td>
<td>NR; died d10</td>
<td>Nf; died</td>
<td></td>
</tr>
<tr>
<td>32 M</td>
<td>Liver, small bowel/21 mo</td>
<td>3</td>
<td>Neg</td>
<td>Jejunum, duodenum, sigmoid colon, rectum</td>
<td>Hyperplastic</td>
<td>ND</td>
<td>RIS</td>
<td>NR; died d25</td>
<td>Nf; died</td>
<td></td>
</tr>
<tr>
<td>33 F</td>
<td>Liver/4 mo</td>
<td>69</td>
<td>Pos</td>
<td>Liver allograft</td>
<td>Hyperplastic</td>
<td>ND</td>
<td>RIS</td>
<td>NR; died d10</td>
<td>Nf; died</td>
<td></td>
</tr>
</tbody>
</table>

Pt indicates patient; Tx, transplantation; y, years; Rx, treatment; F, female; wk, weeks; mo, months; Pos, positive; RIS, reduction of immunosuppression; ACV, aciclovir; CR, complete response; NK, not known; ND, not detected; M, male; LN, lymph nodes; chemo, chemotherapy; radio, radiotherapy; Neg, negative; VCV, valaciclovir; GCV, ganciclovir; PR, partial response; and NR, no response.

*Serum anti-VCA IgG.
†All PTLD tumor biopsies were positive for EBERs by in situ hybridization.
‡Copies of EBV DNA per 10$^6$ PBMCs.
there was an association between the number of matches and patient outcome, with those with a higher number of matches responding better at 6 months than those with fewer matches at 6 months; this reached a statistical significance ($P < 0.048$; Figure 2; Table 2). The response rate was not related to matching or mismatching of any particular HLA antigen locus (A, B, or DR; data not shown).

Better outcome with higher percentage of CD4 cells

All CTLs used for in vivo infusions were polyclonal and contained both CD4+ and CD8+ cell populations, with the percentage of CD3+, CD4+ cells ranging from less than 1% to 60%. At 5 weeks after the first infusion, there was a statistically significant trend ($P = 0.001$) toward better responses in those receiving CTLs with a higher percentage of CD4 cells. Responses for patients receiving CTLs with less than 1%, 1% to 4.9%, and 5% or more CD4 cells were as follows: 3 (25%) of 12, 7 (78%) of 9, and 11 (92%) of 12, respectively (Figure 3). This significant trend was maintained at 6 months with 2 (18%) of 12, 5 (56%) of 9, and 10 (83%) of 12 patients responding, respectively ($P = 0.001$) (Figure 3).

**Figure 1.** The Kaplan-Meier overall survival rate. The values in brackets indicate the number of patients followed up at each time point after the first CTL infusion.

**Figure 2.** Relation between the degree of HLA matching between the patient and CTL donor and clinical outcome. The number of HLA antigen matches between patients and CTL donors showed a trend toward a sustained clinical response at 6 months with greater matching that reached a statistical significance ($P = 0.048$).

**Table 2. Analyses of outcome in association with various parameters**

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>5 wk responders (%)</th>
<th>6 mo responders (%)</th>
<th>$P$</th>
</tr>
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<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>11 (79)</td>
<td>10 (71)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>19</td>
<td>10 (53)</td>
<td>7 (37)</td>
<td></td>
</tr>
<tr>
<td><strong>Age at CTL infusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Younger than 16 y</td>
<td>10</td>
<td>7 (70)</td>
<td>5 (50)</td>
<td></td>
</tr>
<tr>
<td>16 to 49 y</td>
<td>9</td>
<td>6 (67)</td>
<td>5 (56)</td>
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<tr>
<td>50 y or older</td>
<td>14</td>
<td>8 (57)</td>
<td>7 (50)</td>
<td></td>
</tr>
<tr>
<td><strong>Time to PTLD from transplantation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fewer than 2 y</td>
<td>14</td>
<td>9 (64)</td>
<td>9 (64)</td>
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<tr>
<td>2 y or longer</td>
<td>19</td>
<td>12 (63)</td>
<td>8 (42)</td>
<td></td>
</tr>
<tr>
<td><strong>Single vs multiple tumor sites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single site</td>
<td>4</td>
<td>3 (75)</td>
<td>3 (75)</td>
<td></td>
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<tr>
<td>Multiple sites</td>
<td>29</td>
<td>18 (60)</td>
<td>14 (48)</td>
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<tr>
<td><strong>Sites involved</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nodal</td>
<td>11</td>
<td>8 (73)</td>
<td>5 (45)</td>
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<tr>
<td>Extranodal</td>
<td>15</td>
<td>9 (60)</td>
<td>8 (53)</td>
<td></td>
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<tr>
<td>Both</td>
<td>7</td>
<td>4 (57)</td>
<td>4 (57)</td>
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<tr>
<td><strong>Histologic type of PTLD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hyperplastic</td>
<td>5</td>
<td>4 (80)</td>
<td>3 (60)</td>
<td></td>
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<tr>
<td>Hodgkin lymphoma</td>
<td>5</td>
<td>5 (100)</td>
<td>4 (80)</td>
<td></td>
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<tr>
<td>Polymorphic</td>
<td>9</td>
<td>5 (56)</td>
<td>4 (44)</td>
<td></td>
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<tr>
<td>Monomorphic</td>
<td>14</td>
<td>7 (50)</td>
<td>6 (43)</td>
<td></td>
</tr>
<tr>
<td>$P$</td>
<td>—</td>
<td>.23</td>
<td>.999</td>
<td></td>
</tr>
<tr>
<td><strong>Clonality of tumor</strong>*</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyclonal</td>
<td>7</td>
<td>4 (57)</td>
<td>2 (29)</td>
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<tr>
<td>Monoclonal†</td>
<td>10</td>
<td>8 (80)</td>
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<td></td>
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<tr>
<td>Negative</td>
<td>2</td>
<td>2 (100)</td>
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<td></td>
</tr>
<tr>
<td>$P$</td>
<td>—</td>
<td>.001</td>
<td>.001</td>
<td></td>
</tr>
<tr>
<td><strong>Percentage of CD4 cells in infused CTL lines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Less than 1%</td>
<td>13</td>
<td>3 (23)</td>
<td>2 (17)</td>
<td></td>
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<td>1% to 4.9%</td>
<td>9</td>
<td>7 (78)</td>
<td>5 (56)</td>
<td></td>
</tr>
<tr>
<td>5% or more</td>
<td>12</td>
<td>11 (92)</td>
<td>10 (83)</td>
<td></td>
</tr>
<tr>
<td>$P$ trend</td>
<td>—</td>
<td>.001</td>
<td>.001</td>
<td></td>
</tr>
<tr>
<td><strong>Number of HLA matches at HLA A, B, and DR loci</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/6</td>
<td>3</td>
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<td>10</td>
<td>9 (90)</td>
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<td></td>
</tr>
<tr>
<td>$P$ trend</td>
<td>—</td>
<td>.17</td>
<td>.048</td>
<td></td>
</tr>
</tbody>
</table>

*Clonality of tumor not known for 14 patients.
†Includes 5 Hodgkin lymphomas.
5 weeks but later died of cytomegalovirus infection (autopsy showed complete regression of the tumor). The other patient (patient 24) with widespread PTLD involving eye, spine, and lymph nodes did not respond to CTLs.

There was no significant association between response to CTL therapy and histologic type, clonality, or viral gene expression of the PTLD tumors; however, it was notable that 4 (80%) of the 5 hyperplastic tumors and all 5 Hodgkin-type PTLDs responded to CTLs compared with 56% and 50% response rates for polymorphic and monomorphic tumors, respectively (Table 2). The only Burkitt-like tumor showed a complete response that was sustained at 6 months (Table 1).

**EBV viral load in peripheral blood**

EBV DNA was detected in PBMCs of 17 of 33 PTLD patients tested before the first CTL infusion, the viral load ranging from 339 to 1,22 million copies per million PBMCs. Pre-CTL infusion levels of EBV DNA for all 33 patients are shown in Table 1. Four (patients 22, 24, 27, and 31) of 9 patients who had received prior anti-CD20 monoclonal antibody rituximab had detectable EBV DNA in their PBMCs before CTL infusion.

Ten of the 17 patients with preinfusion EBV DNA later showed a complete/partial response to CTL therapy and 7 patients had no response. Viral load fluctuated during the CTL infusions and became undetectable between 1 week to 8 months after the last CTL infusion in all patients (Figure 4A). There was no association between the rate of decrease in viral load and the response to therapy (Figure 4B represents patient 18, PR at 5 weeks, NR at 6 months). Some patients (including 9 who had no detectable DNA before CTL infusion) showed a transient increase in EBV load in PBMCs immediately after CTL infusions (Figure 4C; patient 2 CR).

**Monitoring of infused CTLs**

T-cell receptor (TCR) spectratyping was performed on preinfusion and serial postinfusion PBMCs from 5 patients and on the infused CTLs. The timing of samples tested ranged from 4 hours to 7 days after infusion. The number of monoclonal TCR subfamilies identified in the infused CTLs ranged from 2 to 8 (average number, 4) with no specific pattern of family usage nor any particular subfamily being dominant. Where possible the monoclonal subfamilies identified in the CTLs were traced in the patient PBMC samples. For successful tracing, the CTL monoclonal subfamily signal had to be strong with the same base peak in the pretreatment recipients’ PBMC producing a weak signal. Tracing was successful in 3 of the 5 patients (1 complete responder, 1 partial responder, and 1 nonresponder), with a total of 7 subfamilies traced over the treatment period (2, 4, and 6 infusions). Two different patterns of CTL subfamily trace were observed; a gradual increase in the peak signal with each infusion (2 of 7 subfamilies) and a maximum signal detected at 4 or 24 hours after each infusion (5 of 7 subfamilies; Figure 5A,B, PR and NR, respectively). In one patient, an elevated CTL subfamily could be detected 7 days after the second infusion, but no further samples were available to test.
Monitoring of alloantibody response

Testing of pretransplantation and posttransplantation plasma samples from all trial participants revealed that only one patient had developed an antibody response against a mismatched donor HLA antigen (A2; data not shown).

Discussion

We report the first phase 2 clinical trial using partially HLA-matched allogeneic CTLs for PTLD therapy. Using EBV-specific CTLs grown from peripheral blood of healthy blood donors to treat PTLD on a best HLA-match basis, we show a 52% (17/33) response rate at 6 months. CTL infusions proved simple to administer on an outpatient basis, and no treatment-related acute or long-term toxicity was observed. Thus, since all patients in the trial had failed to respond to conventional PTLD treatments, our response rate of 52% with allogeneic CTLs is encouraging and represents a new, safe, and alternative approach to treating PTLD.

Previous studies using donor-derived EBV-specific CTLs to prevent or treat PTLD in stem cell transplant recipients have reported promising results, but this strategy cannot be used for PTLD in solid organ recipients as donor blood is generally not available. For solid organ recipients, autologous CTLs have been used with some success, but the time and expense required to generate individual CTLs for each patient restricts its wider application. To bypass these restrictions, we developed a CTL bank in Edinburgh that provides a source of EBV-specific CTLs for rapid treatment of PTLD on a best HLA-matched basis.

The primary outcome measure in this trial was tumor response at 6 months as indicated by clinical evaluation in addition to radiologic or MRI scanning where appropriate. Twenty-six of the 33 patients reached the 6-month time point with a response rate (partial or complete) of 52% (17/33). There was a suggestion that response to treatment may be better among female than male patients, but a larger trial is needed to confirm this. If such a difference exists, then the reasons are not clear.

In common with previous studies using conventional PTLD treatment, our results show some indication of a better, sustained response to CTL therapy in early onset disease. Thus, we found that PTLD tumors arising within 2 years of transplantation showed a better response rate at 6 months than those arising at a later time point (64% versus 42%), although the differences were not statistically significant. It is notable that 3 of 4 tumors involving a single anatomic site showed a complete response. At the time of enrollment into our trial, most patients had progressive disease despite having received conventional therapy. In particular, immunosuppression dose reduction was first-line treatment for all patients (Table 1), and for this reason we cannot exclude the possibility that some of the responses we observed were due to the delayed effect of this treatment rather than CTLs. However, we have previously reported complete tumor regression after allogeneic CTL therapy in a child with an EBV-associated brain lymphoma related to a primary immunodeficiency, a situation in which the level of immunosuppression could not be manipulated.

The CTLs used in this study were selected for infusion on a best HLA-match basis, with the number of matches at HLA A, B, and DR loci varying from 2 to 5. We recorded a significant increase \( P = .048 \) in response rate with increased number of HLA matches between CTL donor and recipient, again indicative of the tumor responses being CTL mediated (Figure 2). The CTL lines in the Edinburgh bank were derived from healthy donors and, in order to maintain multiple epitope specificities, were not cloned prior to in vivo use. Most lines consist of CD8 T cells with a minor population of CD4 cells. The outcome of CTL therapy in this trial correlated with the level of CD4 cells within CTLs, with a highly significant improvement in outcome with higher numbers of CD4 cells (Figure 3). This is not an entirely unexpected result as CD4 cells provide help to CD8 cytotoxic T cells, and although most CD8 cells in the CTLs had an effector phenotype ([CD45RO] \(^+\), HLA-DR \(^+\), CD69 \(^+\), CD150 \(^-\)), CD4 cells may have provided additional signals that enhanced their survival in vivo.

Although the timing of the fall in EBV load in peripheral blood that we regularly observed during and following CTL therapy suggests that the CTLs were active in vivo, it is important to note that the rate of fall did not correlate with response to tumor (Figure 4). Thus events in the peripheral blood do not always reflect those in the tissues or tumor sites, and viral load data should be interpreted with caution. Interestingly, in some cases, a transient rise in plasma EBV load occurred immediately after CTL infusions, a phenomenon recorded by others during treatment of PTLD with autologous CTLs and thought to indicate specific lysis of tumor cells.

Heslop et al have previously shown that stem cell transplant donor-derived CTLs survive long term in recipients and can control reactivated persistent EBV infection up to 18 months after infusion. But considering the partial HLA matching and allogeneic nature of the CTLs used in this trial, we expected them to be short-lived in vivo since the mismatched HLA molecules would be targeted by the host immune system. However only one patient developed detectable antialloantibodies during the study, and this individual achieved a complete response. This finding can perhaps be explained by the immunosuppressive therapy that all patients were receiving inhibiting primary immune responses. Using clonotyping to monitor individual T-cell clones within infused CTLs, we demonstrated survival of these cells in peripheral blood for up to 7 days (Figure 5), and in one patient (patient 13) where tetramers
were available to detect individual EBV peptide–specific clones, infused CTLs were detected at 194 days. In this case, the CTLs used contained a high level (34%) of CD4+ T cells, again suggesting that these cells may have prolonged in vivo survival (M.K.G. and D.H.C., data not shown).

Compared with our 52% response rate at 6 months, a recent phase 2 clinical trial using rituximab to treat PTLD reported a response rate of 44% at day 80. Rituximab is available for immediate use and is easy to administer. This study enrolled patients whose only previous treatment was reduction of immunosuppression, and CNS tumors were excluded, whereas, in our trial 50% of patients had received and failed on a variety of therapies in addition to reduction of immunosuppression (Tables 1, 2) and patients with CNS PTLD (n = 5) were included.15 Chemotherapy is commonly preferred for aggressive tumors and gives a comparable response rate to that reported here, however severe toxicity and infections are recurring problems.11,29 Thus there is an urgent need for large multicenter trials comparing and combining these treatment options in order to establish the best treatment practice for patients at all stages of the disease.

In summary, we have shown that partially HLA-matched allogeneic T-cell therapy is a safe and effective option for PTLD and that a CTL bank overcomes the restrictions implicit in autologous CTL therapy. During the trial, frozen CTLs were sent from Edinburgh to France, Sweden, and Australia where they were used to treat PTLD. Thus a single CTL bank could be a valuable international resource.

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Authorship

Contribution: D.H.C. was the principal investigator; D.H.C., T.H., M.T., P.L.A., and A.J.S. were grant holders; T.H. and D.H.C. designed the trial, supervised and trained the team members, liaised with clinicians, provided clinical advice, interpreted and analyzed data, and drafted the paper; G.M.W. was the trial coordinator, liaised with transplantation centers, and maintained databases; G.M.W., M.M.J., G.U., P.W., and D.B. established and maintained CTL bank, analyzed patients’ samples, and performed quality control/sterility checks; M.M.J. also carried out EBV PCR; K.M. performed TCR spectratyping; C.D.H. performed statistical analyses; C.B. provided histologic diagnosis on tumor samples; M.T., P.L.A., and A.J.S. advised on trial design; M.T. coordinated the blood donors; P.L.A. provided clinical advice and phenotyping of cells; D.K., A.M., and M.K.G. enrolled 3 or more PTLD patients to the trial; all the authors read and commented on the final version of the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


Incidence of cytomegalovirus infection among the general population and pregnant women in the United States

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Abstract

Background: Cytomegalovirus (CMV) is a common opportunistic infection among HIV-infected individuals, a major source of serious complications among organ-transplant recipients, and a leading cause of hearing loss, vision loss, and mental retardation among congenitally infected children. Women infected for the first time during pregnancy are especially likely to transmit CMV to their fetuses. More children suffer serious disabilities caused by congenital CMV than by several better-known childhood maladies such as Down syndrome or fetal alcohol syndrome.

Methods: Using CMV seroprevalence data from the nationally representative Third National Health and Nutrition Examination Survey, we estimated CMV incidence among the general United States population and among pregnant women. We employed catalytic models that used age-specific CMV seroprevalences as cumulative markers of past infections in order to derive estimates of three basic parameters: the force of infection, the basic reproductive rate, and the average age of infection. Our main focus was the force of infection, an instantaneous per capita rate of acquisition of infection that approximates the incidence of infection in the seronegative population.

Results: Among the United States population ages 12–49 the force of infection was 1.6 infections per 100 susceptible persons per year (95% confidence interval: 1.2, 2.4). The associated basic reproductive rate of 1.7 indicates that, on average, an infected person transmits CMV to nearly two susceptible people. The average age of CMV infection was 28.6 years. Force of infection was significantly higher among non-Hispanic Blacks (5.7) and Mexican Americans (5.1) than among non-Hispanic Whites (1.4). Force of infection was significantly higher in the low household income group (3.5) than in the middle (2.1) and upper (1.5) household income groups. Based on these CMV incidence estimates, approximately 27,000 new CMV infections occur among seronegative pregnant women in the United States each year.

Conclusion: These thousands of CMV infections in pregnant women, along with the sharp racial/ethnic disparities in CMV incidence, are compelling reasons for accelerating research on vaccines and other interventions for preventing congenital CMV disease. Nevertheless, the relatively low force of infection provides encouraging evidence that modestly effective vaccines and rates of vaccination could significantly reduce CMV transmission.
Background
Cytomegalovirus (CMV) is a common opportunistic infection among human immunodeficiency virus (HIV)-infected individuals, a major source of serious viral complications among organ-transplant recipients, and a leading cause of hearing loss, vision loss, and mental retardation among congenitally infected children. In fact, more children suffer serious disabilities caused by congenital CMV than by several better-known childhood maladies such as Down syndrome or fetal alcohol syndrome [1].

Like other herpesviruses, primary CMV infection is followed by the establishment of lifelong latent infection from which periodic reactivation is common [2,3]. Symptoms are usually absent during primary infection and reactivation, but CMV can be shed in various bodily secretions, particularly urine and saliva [4]. CMV is transmitted person-to-person via close non-sexual contact, sexual activity, breastfeeding, blood transfusions, and organ transplantation [4]. For pregnant women, important sources of infection include sexual activity and contact with the urine or saliva of young children, especially their own children [5-7].

Congenital CMV infection is most likely to occur following a primary infection in the mother during pregnancy [8]. However, maternal CMV reactivation or reinfection with a different CMV strain can also lead to fetal infection [8]. Approximately 10 percent of congenitally infected infants are symptomatic at birth, and of the 90 percent who are asymptomatic, 10–15 percent will develop symptoms over months or even years [9].

Incidence of primary CMV infections has been estimated only in small or specialized populations, such as pregnant women or day care providers. The most comprehensive study of CMV incidence was carried out by Griffiths and colleagues in the United Kingdom [10], in which they estimated that more than three seronegative women per 100 seroconvert each year. However, their study was limited to pregnant women and was hospital-based rather than population-based. Robust, nationally representative estimates of CMV incidence are essential for 1) assessing the burden of primary CMV infection in the United States population, especially among pregnant women; 2) examining whether there are racial/ethnic disparities in primary maternal infection rates, which might be responsible for racial/ethnic disparities in congenital infection rates; and 3) evaluating how effective a vaccine or other intervention must be in order to reduce the incidence of congenital CMV disease. To obtain estimates of CMV incidence in the United States, we employed mathematical models that used age-specific CMV seroprevalences from the Third National Health and Nutrition Examination Survey (NHANES III).

Methods
Study population and design
NHANES III was conducted from 1988 to 1994 and provides nationally representative estimates of the health and nutritional status of the civilian, noninstitutionalized population of the United States. In order to produce population-representative estimates, NHANES III used a multistage, stratified, clustered sample design and generated sample weights proportional to the probability of participant selection. All our analyses used the NHANES III sample weights and sample design variables to correct for population representativeness and the interval estimates for the multistage complex sample design. The study protocol was approved by the authors’ institutional review board. More details about NHANES III can be found in the official documentation [11]. Serologic testing for CMV immunoglobulin G (IgG) was conducted as described previously [12].

The main focus of our models of CMV incidence was the age range 12–49 years. Over 90 percent of participants in this age range had sera available for CMV testing (N = 11,859) so that seroprevalence estimates were representative of the United States population. More importantly, this age range included women of childbearing age and so has key relevance for congenital CMV disease. Although surplus sera was only available for approximately 70 percent of 6–11 year-olds (N = 2,679), we also ran models in this age group to assess whether incidence rates differed by race/ethnicity. Nationally-representative CMV seroprevalence estimates were not available for children less than six years old.

Description of models
Here we give an overview of the models of CMV incidence. A more detailed description is provided in the Appendix. We employed catalytic models [13,14] that used age-specific CMV seroprevalences as cumulative markers of past infections in order to derive estimates of three basic parameters: the force of infection, the basic reproductive rate, and the average age of infection. The force of infection is the instantaneous per capita rate of acquisition of infection [13] and will be expressed in this article as the number of primary CMV infections per 100 seronegative persons per year. The basic reproductive rate is a function of the force of infection and is the average number of secondary infections produced when one infected individual is introduced into a host population where everyone is susceptible. The average age of infection is also a function of the force of infection and is the age at which an individual in a given population typically
acquires a specific infection. We considered parameter differences to be statistically significant when corresponding confidence intervals did not overlap.

Force of infection can be estimated as time-dependent, age-dependent, or both. Since our data were taken from a single, cross-sectional survey, we could not model time dependence. To evaluate age-dependence, we visually inspected the slope of the age-specific seroprevalence graph (see Appendix). We observed no extreme departures from linearity for the overall population, with the slope appearing fairly constant as a function of age. However, because we saw age-dependent changes in slope within some subpopulations (e.g., Figure 1), we used piece-wise log-linear models that allowed the slope to vary between the age groups 6–11, 12–19, and 20–49 years. With the exception of this modification for the subgroup analysis, our final models were the time- and age-independent ones proposed by Griffiths et al. [10] for modeling CMV incidence, where the force of infection is estimated as the slope of the log-linear regression line having the seronegative prevalence as the response variable and age as the explanatory variable. For all models age was treated as a continuous variable.

The models made the following assumptions: CMV infection does not affect the mortality rate; seroprevalence in newborns equals zero; the death rate is type I, meaning everyone survives until a specific age, after which the survival probability is zero; and every person in the population is equally susceptible (i.e., homogeneous mixing).

**Variables**

We estimated the model parameters for the entire United States population and for specific population groups stratified by sex, race/ethnicity, and/or household income. Race/ethnicity was a self-reported variable that consisted of non-Hispanic Whites, non-Hispanic Blacks, Mexican

---

**Figure 1**

Estimating risk of CMV infection during pregnancy

We estimated risk of CMV infection for seronegative women during pregnancy as $\text{risk} = 100 \times [1 - e^{-\text{rate}\times\text{time}}]$, where rate was the force of infection per 100 women per year and time was the duration of pregnancy [16]. We multiplied this risk by the proportion of women who are CMV seronegative to obtain risk of CMV infection during pregnancy for the entire population (i.e., seronegative and seropositive) of women. We then multiplied the risk of infection in the entire population of women by the average number of live-birth pregnancies per year for the years 1988–1994 [17]. This product represented the estimated annual number of women with a primary CMV infection during pregnancy.

Results

The overall force of CMV infection in 12–49 year-olds in the United States was 1.6 per 100 persons per year (Table 1). The associated basic reproductive rate of 1.7 indicates that, on average, an infected person transmits CMV to nearly two susceptible people. The average age of CMV infection was 28.6 years. Among 12–49 year-olds, CMV force of infection was significantly higher among non-Hispanic Blacks (5.7) and Mexican Americans (5.1) than among non-Hispanic Whites (1.4) (Table 1). These differences were reflected in the average age (in years) of infection, which was 16.3 for non-Hispanic Blacks, 17.5 for Mexican Americans, and 29.3 for non-Hispanic Whites. Force of infection was significantly higher in the low household income group (3.5) than in the middle (2.1) and upper (1.5) household income groups.

We observed considerable variation in force of infection when we stratified by age and sex (Figures 1 and 2). Among adolescent girls (ages 12–19 years), non-Hispanic blacks had a substantially higher force of infection (9.9) than the other groups. In contrast, among pre-adolescent girls (ages 6–11 years), Mexican Americans had the highest force of infection (11.0). Among adolescent boys (ages 12–19 years), force of infection was highest in non-Hispanic blacks (6.4) and Mexican Americans (8.7).

Among seronegative women ages 20–49 years, risk of primary CMV infection during a full-term pregnancy was estimated to be 1.38 percent among non-Hispanic Whites, 3.40 percent among non-Hispanic Blacks, and 3.85 percent among Mexican Americans (Table 2). However, among 12–19 year-old seronegative women, risk was much higher for non-Hispanic blacks (7.33 percent) than for Mexican Americans (2.21 percent) and for non-Hispanic whites (0.15 percent). The estimated annual number of women ages 12–49 experiencing primary CMV infection during pregnancy was approximately 27,000. Most of these infections occur in non-Hispanic Whites because they are the largest racial/ethnic group in the U.S. However, non-Hispanic Blacks and Mexican Americans, especially those under age 30, are disproportionately likely to have pregnancies in which they experience primary CMV infections.

Discussion

Robust estimates of the frequency of new CMV infections are essential for understanding and preventing viral transmission. This study provides the first estimates of CMV incidence that are based on population-representative data. We found that among CMV-seronegative individuals aged 12–49 in the United States, nearly one in 60 seroconverts each year.

This relatively low force of infection indicates that CMV is less easily transmitted than some other infections, such as measles or rubella. For these infections, high vaccine efficacy and coverage are required in order to interrupt transmission [18]. In contrast, a CMV vaccine would not need to have such high efficacy and coverage to substantially prevent CMV transmission. Griffiths et al. [10], who estimated forces of CMV infection of 3.1–3.5/100 persons/year in the United Kingdom, showed that modest rates of vaccination (∼60 percent) would be able to eradicate CMV infection from the human population. Our estimates, which are similar but even lower overall (force of infection = 1.6/100 persons/year), provide further evidence that modestly effective vaccines and rates of vaccination could significantly reduce CMV transmission.

Our models identified large racial/ethnic disparities in the frequencies of new CMV infections. The force of infection for CMV was considerably higher in non-Hispanic Blacks and Mexican Americans than in non-Hispanic Whites. The nearly three-fold differences in risk of primary CMV infection among seronegative women could be responsible for much of the racial/ethnic disparities in rates of infants born with congenital CMV [19]. Racial/ethnic differences were especially pronounced among adolescent girls (ages 12–19 years), among whom primary infection was 50 times more likely in seronegative non-Hispanic blacks and 15 times more likely in seronegative Mexican Americans than in non-Hispanic whites. These higher forces of infection (i.e., incidence in seronegative individuals) suggest that CMV is circulating more frequently in these racial/ethnic groups. Thus, seropositive, pregnant non-Hispanic blacks and Mexican Americans may be at a higher risk of suffering re-infection with a different strain.
of CMV, which also places their infants at risk of symptomatic congenital CMV [8]. These disparities indicate that interventions, such as vaccines or education campaigns, may need to be tailored to meet the needs of different racial/ethnic groups and different age groups.

Table 2: Risk and frequency of CMV primary infection during pregnancy in the United States.

<table>
<thead>
<tr>
<th>Ages (years)</th>
<th>% Seronegative</th>
<th>Risk among seronegative women/100 pregnancies*</th>
<th>Risk for all women/100 pregnancies</th>
<th>No. live-birth pregnancies (100's)†</th>
<th>No. women with primary infection during live-birth pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Hispanic White</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12–19</td>
<td>61.0</td>
<td>0.15</td>
<td>0.09</td>
<td>2320</td>
<td>209</td>
</tr>
<tr>
<td>20–29</td>
<td>56.7</td>
<td>1.38</td>
<td>0.78</td>
<td>12140</td>
<td>9469</td>
</tr>
<tr>
<td>30–39</td>
<td>49.4</td>
<td>1.38</td>
<td>0.68</td>
<td>9120</td>
<td>6201</td>
</tr>
<tr>
<td>40–49</td>
<td>38.9</td>
<td>1.38</td>
<td>0.54</td>
<td>510</td>
<td>275</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td></td>
<td></td>
<td>24090</td>
<td>16154</td>
</tr>
<tr>
<td>Non-Hispanic Black</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12–19</td>
<td>42.6</td>
<td>7.33</td>
<td>3.12</td>
<td>1330</td>
<td>4150</td>
</tr>
<tr>
<td>20–29</td>
<td>17.8</td>
<td>3.40</td>
<td>0.61</td>
<td>3060</td>
<td>1867</td>
</tr>
<tr>
<td>30–39</td>
<td>13.4</td>
<td>3.40</td>
<td>0.46</td>
<td>1350</td>
<td>621</td>
</tr>
<tr>
<td>40–49</td>
<td>5.3</td>
<td>3.40</td>
<td>0.18</td>
<td>80</td>
<td>14</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td></td>
<td></td>
<td>5820</td>
<td>6652</td>
</tr>
<tr>
<td>Mexican American</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12–19</td>
<td>30.1</td>
<td>2.21</td>
<td>0.67</td>
<td>1220</td>
<td>817</td>
</tr>
<tr>
<td>20–29</td>
<td>17.5</td>
<td>3.85</td>
<td>0.67</td>
<td>3990</td>
<td>2673</td>
</tr>
<tr>
<td>30–39</td>
<td>10.5</td>
<td>3.85</td>
<td>0.40</td>
<td>1700</td>
<td>680</td>
</tr>
<tr>
<td>40–49</td>
<td>6.8</td>
<td>3.85</td>
<td>0.26</td>
<td>100</td>
<td>26</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td></td>
<td></td>
<td>7010</td>
<td>4196</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>36940</td>
<td>27002</td>
</tr>
</tbody>
</table>

*Number of infections per 100 susceptible persons per year. We considered parameter differences to be statistically significant when corresponding confidence intervals did not overlap. CI, confidence interval.

**Table 1: CMV force of infection, basic reproductive rates, and average age of infection among persons 12–49 years old in the United States.**

<table>
<thead>
<tr>
<th></th>
<th>Force of Infection (95% CI)*</th>
<th>Basic reproductive rate (95% CI)</th>
<th>Average age of infection in years (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entire U.S. population</td>
<td>1.6</td>
<td>1.3–1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.8</td>
<td>1.3–2.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Male</td>
<td>1.5</td>
<td>1.1–1.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Race/Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic Black</td>
<td>5.7</td>
<td>5.1–6.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Mexican American</td>
<td>5.1</td>
<td>4.3–5.6</td>
<td>3.7</td>
</tr>
<tr>
<td>Non-Hispanic White</td>
<td>1.4</td>
<td>1.1–1.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Income per family size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>3.5</td>
<td>2.8–4.5</td>
<td>2.7</td>
</tr>
<tr>
<td>Middle</td>
<td>2.1</td>
<td>1.6–2.6</td>
<td>1.9</td>
</tr>
<tr>
<td>High</td>
<td>1.5</td>
<td>1.1–1.9</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*Number of infections per 100 susceptible persons per year. We considered parameter differences to be statistically significant when corresponding confidence intervals did not overlap. CI, confidence interval.

In addition to race/ethnicity, low household income was a risk factor for CMV infection. People with low household income may be more likely to have a larger family and experience crowding, thus facilitating CMV transmission via close contact. However, because force of infection...
Figure 2

Cytomegalovirus (CMV) force of infection. Cytomegalovirus (CMV) force of infection stratified by sex, age group (6–11, 12–19, and 20–49 years), and race/ethnicity. Circles represent point estimates and lines represent 95% confidence intervals. Negative values for force of infection can occur because the models treat the CMV seroprevalences as if they come from a single cohort followed over time, when in fact they are age-specific seroprevalences of a population at a single point in time. Thus, in the younger ages where the sample sizes are smaller, it is possible for an older age group to have a somewhat lower seroprevalence than a younger age group, which can lead to a negative value for force of infection. We considered force of infection differences to be statistically significant when corresponding confidence intervals did not overlap.
was more strongly associated with race/ethnicity than with household income, high-risk racial/ethnic groups may have a higher prevalence of additional factors related to CMV transmission, such as increased exposure to CMV while caring for young children. A more detailed analysis of risk factors for CMV infection in NHANES III can be found in Staras et al. [12].

Among women ages 20–49 years, force of infection appeared to be independent of age, suggesting that risk of infection during pregnancy is fairly constant during these ages, and that interventions to prevent congenital CMV must target all women of childbearing age. CMV had a higher force of infection than infections transmitted primarily via sex or injection drug use, such as herpes simplex virus type 2 (HSV-2) or hepatitis B virus (HBV). This suggests either that CMV is more easily transmissible through such behaviors [20] or, more likely, that CMV is transmitted via other, additional routes. Given that CMV has been shown to be transmitted via urine or saliva during close, non-sexual contact, it is likely that this sort of transmission plays a major role in the dynamics of CMV infection [7].

We estimated that each year in the United States more than 27,000 pregnant women experience primary CMV infection and are thus at high risk of giving birth to a child with congenital CMV infection. This estimate does not include any fetal losses that may have been caused by primary CMV infection, nor does it include the many pregnancies affected by CMV reactivation or reinfection among seropositive women. The burden of primary CMV infections during pregnancy falls disproportionately on disadvantaged women—those of low income and racial/ethnic minorities. Furthermore, teenaged minority women are at especially high risk of primary CMV infections during pregnancy, due to their high prevalence of susceptibility, high force of infection, and high pregnancy rates.

The risk of primary CMV infection during pregnancy among seronegative women is similar to previous estimates [4]. For seronegative women, CMV infection represents one of the highest risks for fetal damage that they experience during pregnancy [21]. Because CMV transmission is potentially preventable [1], CMV antibody screening prior to or near the beginning of pregnancy should be evaluated as a means of identifying women at high risk for having congenitally infected infants. Studies should pursue whether knowledge of high risk status is a useful motivational tool for modifying behaviors, such as hand hygiene, for reducing risk of infection [22]. Such screening may also lead to the administration of CMV hyperimmunoglobulins or antiviral drugs for prevention or therapy of fetal infection and disease [23,24].

In this study the modeling assumptions appeared to have been reasonably satisfied. On a population level, CMV infection does not contribute significantly to mortality among infected individuals. Nearly all members (=99 percent) of the population are susceptible at birth, and infection is believed to induce life-long immunity. The type I death-rate cut-off was chosen as 70 years to approximate the U.S. life expectancy during the years that NHANES III was conducted, but modifying the cut-off had little effect on the model results. The assumption of homogeneous mixing is unlikely to be completely true, but because CMV infection is common and has multiple transmission modes, susceptible individuals are likely to have similar risks of exposure to CMV.

An important limitation of our models was that the data were from a single, cross-sectional study so that time trends were not able to be addressed. Thus, high CMV seroprevalence in cohorts of older people might not reflect current incidence and could cause the models to overestimate the force of infection [12]. We sought to minimize this potential bias by focusing most of our analyses on a limited age range (12–49 years). It is also important to note that our younger, age-specific force of infection estimates (i.e., for ages 6–11 and 12–19 years) were imprecise, with wide confidence intervals. Furthermore, the models implicitly assumed that seroprevalence was monotonically increasing with age, as if this cross-sectional study were a cohort study in which seroprevalence was measured at various ages of follow-up. However, this assumption was violated for some of the younger subpopulations. As a result, we occasionally obtained negative estimates for the force of infection (Figure 2), although these estimates were not statistically different from zero.

The calculations of risk of primary infection during pregnancy required several assumptions, one of which was that the force of infection was the same for pregnant and non-pregnant women. Women who are pregnant may have fewer sex partners (and thus lower risk of exposure to CMV) during pregnancy; on the other hand, pregnant women may be more likely than non-pregnant women to be exposed to young children (a group that frequently sheds CMV). Pregnant women may also have a higher risk of acquiring infections because of pregnancy-induced immune depression [25].

Based on our models, we would estimate that more than one million United States women have experienced primary CMV infections during pregnancy since CMV was first isolated 50 years ago [26,27]. A substantial proportion of these infections would have led to congenital infections, leaving thousands of children with lifelong disabilities. Children from disadvantaged racial/ethnic groups are likely to have been disproportionately
impacted. These many affected children are a compelling argument for accelerating research on vaccines and other interventions for the prevention of congenital CMV [28].

Conclusion
Each year, thousands of CMV infections occur in pregnant women in the United States, putting numerous unborn babies at risk for serious disabilities. Incidence of CMV infection in pregnant women is not distributed evenly, but exhibits sharp racial/ethnic disparities, especially affecting non-Hispanic blacks and Mexican Americans. Because of the magnitude of the problem and its associated health disparities, there is an urgent need to accelerate research on vaccines and other interventions for preventing congenital CMV disease. Nevertheless, the low incidence of CMV infection relative to other vaccine-preventable infections provides encouraging evidence that

![Example of piece-wise log-linear model among non-Hispanic black women.](image)

Table 3: Comparison of force of infection for different viruses for selected* age ranges.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Force of infection (per 100 persons per year)</th>
<th>Ages modeled</th>
<th>Study sample</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measles</td>
<td>20</td>
<td>11–17</td>
<td>Lit. review – misc. sources</td>
<td>[18]</td>
</tr>
<tr>
<td>Mumps</td>
<td>12</td>
<td>11–17</td>
<td>Lit. review – misc. sources</td>
<td>[18]</td>
</tr>
<tr>
<td>Rubella</td>
<td>10</td>
<td>11–17</td>
<td>Lit. review – misc. sources</td>
<td>[18]</td>
</tr>
<tr>
<td>Varicella</td>
<td>6</td>
<td>≥ 10</td>
<td>Convenience sample</td>
<td>[30]</td>
</tr>
<tr>
<td>CMV†</td>
<td>3.1 and 3.5</td>
<td>16–40</td>
<td>Hospital-based</td>
<td>[10]</td>
</tr>
<tr>
<td>CMV</td>
<td>1.8</td>
<td>12–49</td>
<td>Population-based</td>
<td>Current study</td>
</tr>
<tr>
<td>HSV-2</td>
<td>0.84</td>
<td>≥ 12</td>
<td>Population-based</td>
<td>[31]</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>0.15</td>
<td>6–39</td>
<td>Population-based</td>
<td>[32]</td>
</tr>
</tbody>
</table>

*Ages were selected to be roughly comparable with the ages we modeled; in general, young children were not selected for comparison because they often had much higher forces of infection. †Patients were recruited from 2 different hospitals.
modestly effective vaccines and rates of vaccination could significantly reduce CMV transmission.

Competing interests
The author(s) declare that they have no competing interests.

Authors’ contributions
FABC designed and carried out the mathematical modeling and statistical analyses and drafted the manuscript. SASS participated in the design and implementation of the CMV testing of the NHANES III specimens. SCD coordinated and supervised the CMV testing of the NHANES III specimens. MIC conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript and revised it critically for important intellectual content.

Appendix
To estimate CMV incidence by using the force of infection, we used the catalytic model approach described in Farrington [14] and Anderson [13]. We began by assuming that the force of infection was age-dependent, so that

\[
S^{-}(a) = e^{-\int_0^a \lambda(x) dx},
\]

where \(a\) is age and \(S(a)\) is the age distribution for the seronegatives. To assess the shape of the integral above we proceeded as Farrington, by visual inspections. \(\lambda(x)\) was evaluated as an exponential decay function and as a polynomial of third or lesser degree. Despite permitting \(\lambda(x)\) to be a complicated function, force of infection was approximately constant as a function of age (i.e., force of infection was age-independent). Therefore, we used the log-linear approach where the force of infection is the slope of the regression model (i.e., log-linear approach where the force of infection is approximately constant as a function of age (i.e., force of infection, \(\lambda\)). We proceeded as Farrington, by visual inspections.

When estimating force of infection for different subgroup categories, one category was chosen to be the referent category and the others were represented by indicator variables and were included in the models with interaction for age. For example, in the case of race/ethnicity, which had 3 categories and White as the referent category, the model was:

\[
\ln(S(a)) = -(\beta_0 + \lambda_0 a + \beta_1 \delta_{\text{Black}} + \beta_2 \delta_{\text{Mexican}} + \lambda_1 \delta_{\text{Black}} + \lambda_2 \delta_{\text{Mexican}} a),
\]

where \(\delta[X] = 1\) if \(X\) and 0 otherwise.

The final models were estimated using the STATA 8.0 (College Station, TX) svypoisson command (log-linear model), which is appropriate for complex survey estimation. The sample weight, cluster, and strata variables suggested by the NHANES III analytical guidelines were used to adjust the estimates for the sample design. The variance was estimated by the linearization method [29]. The \(R_0\) and \(A\) and their confidence intervals were estimated using the nlcom command for non-linear transformations of the regression parameters.

Acknowledgements
We thank Kay Radford, Ashley Schoenfisch and Minal Amin for serologic testing and Geraldine McQuillan for technical assistance. This study was supported in part by funding from the National Vaccine Program Office. Fernando Colugnati received support from the Brazilian Centro Nacional de Pesquisa while he was a guest researcher at the CDC. This research was supported in part by an appointment to the Research Participation Program at the Centers for Disease Control and Prevention administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and CDC (stipend support for Stephanie A.S. Starrs).

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