High-dose interferon-alfa2a exerts potent activity against human immunodeficiency virus type 1 not associated with antitumor activity in subjects with Kaposi's sarcoma


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Characterization of the T Cell Response to Human Rhinovirus in Children: Implications for Understanding the Immunopathology of the Common Cold

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Human rhinovirus (HRV) is a frequent respiratory pathogen, responsible for a large proportion of cases of the “common cold” and linked to acute asthma, especially in children. T cell responses to HRV and their contribution to HRV-associated pathology were investigated. T cells were obtained from tonsils removed from children at routine tonsillectomy. Proliferative and cytokine responses were measured after in vitro restimulation with purified HRV preparations of both major and minor serotypes. Most tonsils tested showed T cell proliferation, and responses to multiple serotypes in one tonsil were observed frequently. Responding T cells were CD4-positive and produced interleukin-2 and interferon-γ but no interleukin-4. Thus, children respond to HRV, a proportion of the response is serotype-cross-reactive, and a Th1 reaction predominates. In addition to contributing to protection, this response may enhance expression of virus receptor and be implicated in the immunopathology of HRV infection.

Human rhinovirus (HRV) is the most common cause of upper respiratory tract disease in humans. Although HRV seldom leads to serious illness, infection is associated with considerable loss of working time in developed countries and with other acute respiratory illness, especially exacerbations of allergic asthma, in both children and adults. Therefore, there is a need for better understanding of the immunopathology of HRV infection and of the molecular processes that link this immunopathology with other forms of pulmonary disease [1–3].

Like other picornaviruses, HRV induces serotype-specific neutralizing antibodies in both humans and experimental animals, usually recognizing only 1 of >100 known serotypes. The specificity and slowness of response, detectable in nasal secretions 2–3 weeks after infection when virus is cleared from the upper respiratory tract [4, 5], has made it difficult to devise a strategy to stimulate cross-reactive antibodies of protective value in the general population. The implication of this delay is that antibody probably plays little role in the early stages of HRV infection. Local cytokine production (e.g., interferon [IFN]) is likely to play the more dominant role during this period, although reactivation of a memory T cell response could also be a component.

Previous studies have characterized the murine T cell response to HRV [6]. HRV stimulated T cell proliferation and interleukin (IL)-2 production. In contrast to antibody, the T cell response was directed largely at internal capsid epitopes common to many serotypes. This suggested that HRV may be able to stimulate cross-reactive Th1-type responses, with implications in the natural history of the viral infection as well as in the pathogenesis of the other forms of respiratory disease. The conclusions of the murine studies were, however, limited; HRV does not produce productive infection in rodents [7].

In this study, the human T cell proliferative and cytokine responses to a variety of serotypes have been analyzed by use of human tonsil, a local nasopharyngeal mucosal lymphoid tissue where HRV infection first manifests. The cytokine spectrum demonstrated may be important not only in the immunopathology of HRV infection but also in the respiratory complications with which it has been associated.

Materials and Methods

Preparation of HRV. Methods for viral growth and purification have been described previously [6]. Virus was purified from HeLa-derived epithelial cell lysates, fractionated through a 15%–45% continuous sucrose gradient, and then detected by ELISA with HRV serotype–specific rabbit antisera. HRV-15 (major serotype) and HRV-1A or HRV-2 (two closely related members of the minor serotypes, chosen because of information available on structure) [8] were used unless otherwise indicated.

Isolation of cells from human tonsils. Tonsils obtained immediately after tonsillectomy for routine clinical indications (and in the absence of acute infection) from children between 3 and 14 years old were washed (70% ethanol), cut into 1-mm pieces, and digested (collagenase, 1 mg/mL, for 60 min at 37°C; Sigma, Poole, UK; in Hanks’ balanced salt solution [HBSS]). Digestion was
stopped with RPMI–5% fetal calf serum (FCS), and the suspension was forced through sterile 125-μm-pore nylon mesh. Cells were washed, resuspended in 10× HBSS—isotonic Percoll, and fractionated by flotation through a Percoll gradient (800 g, 30 min). Low-density cells (those found at the interface between 30% and 40% and between 40% and 50% Percoll) and high-density cells (50%–60% and 60%–70% interface) were collected, washed, and cultured overnight (5 × 10^6/mL in RPMI with 10% FCS, 10 mM HEPES, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B [complete medium]). Media and supplements were from Life Technologies (Paisley, UK).

Enriched resting T cells were from high-density 24-h nonadherent cells (CD3, 78%; CD4, 31%; CD8, 25%) either by E-rosetting or by depletion of B cells (CD19), macrophages (CD14), and HLA-DR cells, by use of second-layer Dynabeads and rabbit anti-mouse immunoglobulin. To generate >95% CD3 T cells, low-density cells were CD3-depleted, and the remaining cells (75% CD19 B cells, and dendritic cells) were irradiated (3000 Rad, cobalt irradiation source) and used as antigen-presenting cells (APC). For CD4 and CD8 cell depletion, high-density T cells were depleted of HLA-DR, CD19, and either CD4 or CD8 cells and the required population was isolated by negative selection by use of Dynabeads (as above). Purity of the resultant populations was confirmed by flow cytometry (CD4-depleted cells: <1% CD4 cells; CD8-depleted cells: <2% CD8 cells).

**Antibodies.** Antibodies used were CD19 (BU12; gift of D. Hardie, Birmingham University, Birmingham, UK) and HLA-DR (L243), CD4 (QS4120), CD8 (UCHT4), and CD3 (UCHT1) (gifts of P. Beverley, University College London, London).

**Proliferation assays.** T cells (4 × 10^5/well) were cultured with irradiated APC (10^5/well) and 4.0 μg/mL HRV (i.e., ~10^6 virus particles/well) or 2.5 μg/mL concanavalin A (ConA; Sigma) for 7 days in flat-bottom microtiter plates (200 μL) of complete medium at 37°C, humidified 5% CO2 incubator, as based on preliminary experiments testing a range of T cell, APC, and virus concentrations and incubation times. Proliferation was measured by use of 1 μCi/well [3H]thymidine for the final 16 h. Mean ± SD of radiolabel incorporation was calculated from triplicate wells. For some experiments, results are shown as ratio of mean incorporation in the presence and absence of virus (stimulation index [SI]).

**Cytokine assays.** Supernatants were collected and stored at −20°C. IL-4 and IFN-γ were tested by ELISA (Genzyme, Cambridge, MA). IL-2 was tested by use of a CTLL bioassay; ELISA confirmed that under these conditions, the bioassay correlated with supernatant IL-2, not with other cytokines.

**Results**

Initial experiments demonstrated an HRV-specific, dose-dependent response in T cells isolated from tonsil (data not shown). The optimal dose in preliminary experiments, 4 μg/mL, was used routinely. No response was detected when “mock” HRV from HeLa cultures lysed and purified in the same way was used (data not shown).

Figure 1A summarizes SIs when T cells plus APC from a series of 34 unselected tonsils were cultured with major (HRV-15) and minor (HRV-1A or HRV-2) forms of the virus. Of 34 tonsils tested, 27 (75%) responded (SI >2) to at least 1 serotype, with no significant difference between major and minor serotypes (average SI = 4.2 ± 2.9 and 3.9 ± 2.2), although 7 of 8 responses with SIs >9 were to the major serotype. Correlation between responses to major and minor serotypes (figure 1B) shows that in general, nonresponders responded to neither serotype (7/34), although ConA responses were normal. Overall, responses to major and minor serotypes correlated (r = .569, P < .001), although individual tonsils showing a stronger response to one virus than to another were observed.

In a proportion of randomly selected tonsils (8/34), responses to a wider panel of serotypes were tested. Individual variations in relative SI were seen, but the results shown in figure 1C are a representative example: 6 of 8 tonsils responded to all 4 serotypes with SIs > 30% and 40% and between 40% and 50% Percoll) and high-density cells (50%–60% and 60%–70% interface) were collected, washed, and cultured overnight (5 × 10^6/mL in RPMI with 10% FCS, 10 mM HEPES, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B [complete medium]). Media and supplements were from Life Technologies (Paisley, UK).

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In a proportion of randomly selected tonsils (8/34), responses to a wider panel of serotypes were tested. Individual variations in relative SI were seen, but the results shown in figure 1C are a representative example: 6 of 8 tonsils responded to all 4 major HRV serotypes and 3 minor serotypes. As previously, T cells in 2 tonsils failed to respond to any HRV serotype, although responses to ConA were normal.

Infectious virus may load both class I and class II antigen-processing pathways, so in four further experiments, the phenotype of the responding T cells was examined by depletion of either CD4 or CD8 cells. CD4 cell depletion inhibited proliferation (e.g., APC + CD4 cell–depleted T cells: <4 × 10^5 cpm of [3H]thymidine with both HRV-1A and HRV-15), whereas in the same assay, depletion of CD8 cells had little effect (e.g., APC + CD8 cell–depleted T cells: >3 × 10^4 cpm of [3H]thymidine with HRV-1A and HRV-15; unfractionated T cells: 3.5 × 10^5 cpm of [3H]thymidine) (SD <5%). Responder phenotyping showed increased numbers of CD4 cells at the end of the assay (data not shown), and depletion of HLA-DR–positive APC completely abolished the response to HRV (e.g., HLA-DR cell–depleted T cells + HRV-15: <2 × 10^5 cpm of [3H]thymidine; HLA-DR–positive APC + HRV-15: <1.8 × 10^4 cpm of [3H]thymidine). Furthermore, UV-inactivated virus, unable to infect target cells, stimulated proliferation equivalent to that seen with live virus (data not shown), implying that intracellular production of viral protein was not required.

The cytokine profile of responding T cells stimulated with HRV-15 or HRV-1A is summarized in figure 2A. HRV proliferation always correlated with IL-2 and IFN-γ release, but no IL-4 (i.e., <10 pg/mL) was detected, in either proliferating or nonproliferating cultures. Figure 2B documents this further: IL-2 concentrations were maximal after 24 h, then declined rapidly; IFN-γ levels peaked at day 3 and remained high throughout the culture period.

**Discussion**

Although HRV is a common pathogen in humans, few studies have addressed cell-mediated responses to HRV. Therefore, T cell responses to HRV (both major and minor serotypes) were examined, by use of cells from tonsil, and show clearly that such responses can be demonstrated.
Figure 1. Proliferative responses to HRV in tonsillar T cells. A. Stimulation index (ratio of thymidine incorporation in presence or absence of antigen), plotted as no. of tonsils vs. range of stimulation index (i.e., frequency histogram). Results using HRV-1A and -2 were combined since these 2 serotypes are structurally very similar [7], and responses showed no significant difference between them. B. Stimulation index of response to HRV-15 in 34 tonsils plotted vs. stimulation index to HRV-1A. Similar results were obtained for HRV-2. Line shows regression analysis ($r = .569; P < .001$). C. Representative example of response of tonsillar T cells from 1 patient to panel of HRV serotypes (stimulation indices). Similar results were obtained for 6 of 8 patients tested. HRV-1A, -2, and -29 are minor serotypes, while HRV-3, -7, -14, and -15 belong to major serotype group.

One potential disadvantage of using tonsillar T cells is that surgery is performed after repeated upper respiratory tract infections, raising the possibility that these tonsils are not representative. Serotype-specific proliferative responses to rhinovirus have been found in peripheral blood mononuclear cells after, but not before, priming in vivo by infection [9], and the same may apply in the tonsil. Nevertheless, the frequency of the response observed here was surprising, suggesting that most persons have responded to HRV exposure in vivo by priming a T cell antigen-specific response.

The results do not exclude HRV stimulation of primary T cell responses in vitro, or HRV acting as a superantigen, and these
Figure 2. Cytokine production (interleukin-2 [IL-2] and interferon-γ [IFN-γ]) by T cells stimulated with HRV-1A and HRV-15. A, Cytokine production in cultures with T cells, antigen-presenting cells, and either medium alone, HRV-1A, or HRV-15. Data are mean ± SE for 7 tonsils; ranges are in parentheses above bars. Comparison of cytokine release in presence or absence of virus, by use of paired Student’s t test: P < .04 (HRV-1A, IL-2), P < .02 (HRV-15, IL-2), P < .008 (HRV-1A, IFN-γ), P < .01 (HRV-15, IFN-γ). B, Time course of cytokine release. Data from 1 representative experiment of 7 summarized in A are shown.

... possibilities are being explored, but preliminary work in our laboratory suggests that they are unlikely. The study, however, does clarify that in humans, as in mice, HRV is immunogenic with respect to T cells and that immunogenicity in vitro does not depend on infectivity, since inactivated virus induced similar responses. Furthermore, a proportion of these T cells may be directed at serotype–cross-reactive epitopes, since responses were either to the panel of 7 serotypes or to no virus. Future studies looking at clonal analysis will therefore be of interest.

Although the presence of a CD8 T cell response to HRV is not excluded here, and such CD8 cells will undoubtedly play a role in the elimination of infected cells, the data reported here relate primarily to CD4 cells. Responses to HRV were predominantly Th1 CD4 type, abolished by CD4 but not CD8 depletion and by removal of HLA-DR–expressing APC. Furthermore, unexpectedly for a mucosal infection, supernatants from HRV-stimulated T cells contain IL-2 and IFN-γ but no detectable IL-4. Trace undetectable IL-4 might be produced, but the balance of the cytokine profile is clearly tilted toward Th1. This is consistent with previous HRV work using peripheral blood T cells (rather than tonsil) and looking at experimental infection rather than natural history [10].

Production of Th1 cytokines in response to HRV has implications for understanding the relationship between HRV infection and host immune responses. Antigen-specific anti-HRV secretory IgA can be both neutralizing and protective against future challenge with cross-reactive virus [5]. Th1 cytokines (such as IFN-γ) may antagonize this by inhibiting the humoral responses [11] and delaying induction of neutralizing antibody [12]. On the other hand, IFN-γ may also contribute to virus clearance by increasing cellular cytotoxic responses.

Release of Th1 cytokines may not only modulate host capacity to mount effective responses to HRV but also participate in the immunopathology of HRV infection and, in particular, in linking HRV infection and acute asthma [2, 3]. In this context, IFN-γ and other Th1 cytokines can up-regulate expression...
of CD54 (the major HRV receptor) and stimulate release of inflammatory mediators and intercines from both epithelial cells [13] and macrophages, which can then act either alone or in synergy with common microorganisms. Thus, Th1 cytokines can cause strong local inflammation with little protective value but with potential for considerable local damage to host tissues.

Thus, these studies contribute to the hypothesis that HRV-associated pathology may not be due directly to viral cytopathic effects but rather to inappropriate stimulation of local inflammation. This is consistent with clinical observations that exogenous IFN-γ enhances symptoms of HRV infection, with no protective role [14]; with restriction of viral replication to few epithelial cells [15]; with association of HRV infection and lymphocytic or granulocytic infiltrates in the bronchial submucosa [3]; and with infection of epithelial cells stimulating cytokine production [13]. Under normal conditions, this will be self-limiting, resulting in mild symptoms of the common cold, but where the airways have been sensitized (e.g., in chronic allergy), HRV-induced immunopathologic responses may trigger serious “bystander” secondary symptoms, as in acute asthma. Therefore, immunologic intervention in HRV infection aiming to alleviate complications should perhaps be targeted toward switching the cytokine balance of the host response.

Acknowledgments

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References

Outbreak of Adenovirus 35 Pneumonia among Adult Residents and Staff of a Chronic Care Psychiatric Facility

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Division of Field Epidemiology, Epidemiology Program Office, and Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; Rhode Island Department of Health, Division of Disease Control, Providence, Rhode Island

Outbreaks of acute respiratory disease caused by adenovirus are rarely documented in civilian populations, and adenovirus 35 is an uncommon serotype best recognized as a cause of serious disease in immunocompromised patients. An outbreak of adenovirus 35 pneumonia among residents and staff of a chronic care psychiatric facility was investigated. Fourteen (26%) of 53 residents and 4 (2%) of ~200 staff had radiographically confirmed pneumonia. Thirteen (93%) of 14 residents with pneumonia were hospitalized, 5 (36%) required mechanical ventilation, and 1 (7%) died. One staff member was hospitalized. Adenovirus infection was diagnosed in 17 (94%) persons with pneumonia by culture or serology and was confirmed as adenovirus 35 infection in 8 persons. Residents with pneumonia had resided at the facility longer than other residents. Chronic illness was not a risk factor for severe disease. Crowding and poor hygienic behaviors probably facilitated transmission among residents.

In adults, pneumonia is an uncommon but potentially life-threatening complication of adenovirus infection, especially in the immunocompromised host [1, 2]. Adenovirus 35 (Ad35) was first identified in 1975 in renal transplant patients [3], and subsequently it has been recognized as an important respiratory tract pathogen in other immunocompromised patients, especially patients with AIDS [2, 4]. Ad35 is much less commonly identified in the immunocompetent host, and outbreaks of Ad35 disease have not been reported previously. On 18 July 1995, 3 adult residents from the psychiatric building of a chronic care hospital were admitted to a medical intensive care unit with Ad35 pneumonia. This report describes our investigation of the outbreak.

Methods

Epidemiologic investigation. Medical records of facility residents were reviewed and the professional staff was interviewed to identify patients with recent febrile or respiratory illnesses. Potential risk factors, including current medications, underlying medical conditions, length of institutional confinement, behavior, social contacts, and travel outside the facility, were recorded using a standard form. Active surveillance was initiated to identify new cases. Paired sera and nasopharyngeal swabs or sputum were collected from all residents. Additional specimens (e.g., lung biopsy samples or bronchial washings) were available for laboratory testing from intubated patients. All employees of the affected facility and staff who had provided care to ill residents at a medical facility located in another building on the hospital campus were invited to participate in the study and asked to complete a questionnaire and submit serum samples and a nasopharyngeal swab.

Laboratory investigation. Respiratory specimens were inoculated into A549 cells and monitored for cytopathic effect. Cytopathic agents identified as adenovirus by direct fluorescent antibody were submitted to the Centers for Disease Control and Prevention (CDC) for typing. The adenovirus subgenus was determined by polymerase chain reaction assay (PCR) [5], and the serotype was identified by hemagglutination inhibition and micro-neutralization assays using adenovirus type–specific horse antisera [6]. Serum specimens were tested for adenovirus group–specific IgG antibodies by EIA [7]. A positive test result was defined as a ≥4-fold rise or decline in antibody titer between paired serum samples. Additional microbiologic and serologic tests were performed on all ill patients, including antibody testing for Legionella, Mycoplasma, Histoplasma, and Chlamydia species, hantavirus, and other infectious agents.

Results

Patient investigation. During the outbreak, 14 (26%) of 53 residents of the chronic care psychiatric facility had radiographically confirmed pneumonia, 24 (45%) had an acute illness with cough, fever, coryza, otitis, sinusitis, or bronchitis, and 15 (28%) remained asymptomatic. The mean age of residents with pneumonia was 37 years (range, 23–52), compared with...
Table 1. Adenovirus test results for hospital residents and employees by illness category.

<table>
<thead>
<tr>
<th>Group, illness category</th>
<th>Culture</th>
<th>EIA serology*</th>
<th>Laboratory-confirmed, any test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>11/14 (79)</td>
<td>7/14 (50)</td>
<td>13/14 (93)</td>
</tr>
<tr>
<td>Fever or other respiratory signs/symptoms</td>
<td>1/24 (4)</td>
<td>1/24 (4)</td>
<td>2/24 (8)</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>0/15 (0)</td>
<td>2/15 (13)</td>
<td>2/15 (13)</td>
</tr>
<tr>
<td>Total</td>
<td>12/53 (23)</td>
<td>10/53 (19)</td>
<td>17/53 (32)</td>
</tr>
<tr>
<td>Employees</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>2/4 (50)</td>
<td>3/4 (75)</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>Fever or other respiratory signs/symptoms</td>
<td>1/5 (20)</td>
<td>2/10 (20)</td>
<td>3/14 (21)</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>0/1 (0)</td>
<td>0/10 (0)</td>
<td>0/15 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>3/10 (30)</td>
<td>5/24 (21)</td>
<td>7/33 (21)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. positive/no. tested (%).
* No. of persons with ≥4-fold rise in antibody titer with paired sera; includes 2 ill persons with high initial titers and ≥4-fold decline.

42 years for other residents. Four residents with pneumonia were women, and attack rates did not differ significantly by sex. Laboratory test results confirmed adenovirus infection in 17 (32%) residents, including 13 with pneumonia, 2 with fever or respiratory symptoms, and 2 without symptoms (table 1).

The index case was a resident who had onset of pneumonia on 20 June 1995 and may have been the source of the outbreak (figure 1). He was transferred from a short-term care psychiatric hospital just 6 days before onset of symptoms. When his serum was tested 5 weeks after symptom onset, the titer was positive at ≥3200; it fell to 200 on repeat testing 3 months later. The last person with pneumonia had onset of illness on 9 August 1995. In all, 13 (93%) of the 14 residents with pneumonia were hospitalized, 5 (36%) required mechanical ventilation and treatment for hypotension, and 1 patient with chronic renal failure maintained on hemodialysis died while still intubated.

The facility housed the 53 residents on three floors in one building, with 15–19 residents on each floor. Cases of pneumonia were not evenly distributed by floor. The highest attack rate was 53% (10/19), compared with 11% (2/19) and 13% (2/15) on the other two floors (Fisher’s exact test, two-tailed; \( P < .05 \)). However, the index case was not on the floor with the highest attack rate. Most rooms housed 2–5 residents, but no clustering was apparent by room on the floor with the highest attack rate.

Residents with pneumonia had resided at the facility longer (median, 5.7 years) than other residents (median, 2.8 years) (Wilcoxon rank sign test; \( P = .052 \)), and the difference was greatest on the floor with the largest number of cases.

Clozapine, an antipsychotic agent associated with agranulocytosis, was used by 7 (50%) of the 14 residents with pneumonia and 13 (33%) of the other 39 residents. However, none of the patients taking clozapine had a history of significant neutropenia before their illness, and no statistical association was found between its use and adenovirus infection. Hospital policy required that all residents using clozapine be screened weekly for neutropenia. Clozapine was discontinued for all residents admitted to the intensive care unit because of its potential bone marrow toxicity. Several of the residents with adenovirus pneumonia had decreased white blood cell counts related to their sepsis.

The prevalence of smoking (86%, 12/14) among pneumonia patients was similar to that (82%, 32/39) for the other residents. Exposure to secondhand smoke was unavoidable. Although 5 of the 14 residents with pneumonia had underlying chronic illnesses (i.e., chronic obstructive pulmonary disease, asthma, lobectomy for lung adenocarcinoma, diabetes mellitus, chronic renal failure, hepatitis C, or a seizure disorder), having a chronic illness was not statistically associated with developing adenovirus pneumonia.

Residents of the facility were ambulatory and spent the majority of their time in a large common room located centrally on each floor, in the smoking room, or outside. Before the outbreak, residents from all three floors frequently would mingle during planned activities inside or to smoke and socialize outside when weather permitted. Personal hygiene and hand-washing practices were poor. Behaviors that may have facilitated transmission of adenovirus, such as drooling, spitting, and sharing cigarettes, soda cans, or eating utensils, were commonly observed among residents. Illness was not restricted to particular resident social groups.

Staff investigation. More than 200 employees were exposed to ill residents; 33 participated in the study, including

Figure 1. Cases of radiographically confirmed pneumonia by floor and date of illness onset, chronic care psychiatric facility, 1995.
all known to be ill with pneumonia. The participants included 31 employees from the affected facility and 2 staff who cared for ill residents at a medical facility located on the same hospital campus as the psychiatric building. Four (2%) staff had confirmed adenovirus pneumonia; 1 was hospitalized, and none died. The mean age of staff with pneumonia was 47 years. Adenovirus was recovered from 3 (30%) of 10 employees tested, 9 of whom reported recent respiratory symptoms, and serotyping confirmed Ad35 for the 2 isolates for which it was done. Paired sera were collected from 24 employees, 5 (21%) of whom had a 4-fold rise in their antibody titer. Many additional staff reported respiratory symptoms but declined to have serologic testing or culturing done.

Laboratory investigation. Of the 18 original adenovirus isolates from 13 persons, 13 were submitted to CDC for typing. One isolate could not be regrown in cell culture or detected by PCR; the remaining 12 isolates were identified as adenovirus subgenus B by PCR. Of these, 10 isolates from 8 persons (5 residents and 3 staff) were serotyped unambiguously as Ad35, and 2 isolates from residents were cross-reactive by both hemagglutination inhibition and neutralization assays for both Ad35 and Ad21.

The bronchoalveolar lavage specimen from 1 resident with adenovirus pneumonia was also culture-positive for Mycobacterium tuberculosis. This resident had no radiographic evidence of cavitary disease. A contact investigation revealed no additional cases of active tuberculosis. No other pathogens were identified among the remaining ill residents and staff.

Discussion

Since its discovery in 1975, Ad35 has been closely associated with severe illness only in immunocompromised patients [2]. An outbreak of pneumonia caused by Ad35 has not been previously documented, nor has serious respiratory tract disease due to Ad35 been reported in the literature among immunocompetent adults. Our investigation identified several factors that may have contributed to this unusual outbreak.

The observation that residents with pneumonia had been institutionalized for twice as long, on average, as those residents who did not have pneumonia suggests that susceptibility to severe disease may have been increased because of waning immunity as a result of prolonged hospitalization and infrequent exposure to Ad35. However, once Ad35 was introduced, group living conditions combined with poor hygienic behaviors (e.g., limited handwashing, coughing without covering the mouth, spitting, drooling, and sharing cigarettes, drinks, and eating utensils) among residents could explain the explosive propagation among residents, as well as explain why attack rates were so much lower for staff than for residents.

While we were not able to demonstrate a positive association between smoking and severe disease, we still suspect that smoking was an important contributing factor in this outbreak. The lack of a statistically significant association may be due to the small numbers in our cohort and the high prevalence of smoking among residents. Because Ad35 has been linked most often with immunocompromised patients, we were suspicious early on that clozapine, an antipsychotic agent associated with agranulocytosis and used by many of the residents, was a risk factor [8, 9]. Residents taking the medication were monitored weekly, but none had significant neutropenia requiring discontinuation prior to onset of illness.

While control measures are difficult to enforce in a population of this kind because of a lack of patient understanding and compliance, environmental decontamination and patient isolation or cohorting should be attempted. The current adenovirus vaccine is not available for civilian use, does not contain Ad35, and would not have been useful in preventing or controlling this outbreak [10]. We suspect that adenovirus often is overlooked as an etiologic agent of outbreaks of acute respiratory illness but should be considered. It is possible that outbreaks such as the one we report are more common than we suspect.

Acknowledgments

We thank Helen Cai to and Uptala Bandy for assisting in the field investigation, Laura Fehrs for guidance during the field investigation, Priscilla McDermott and the Rhode Island Hospital Virology Laboratory for performing virus isolations, Barbara C. Anderson for performing adenovirus serologic tests, Michael McDonough for performing PCR assays, Larry J. Anderson for review of the manuscript, and John O’Connor for editorial assistance.

References

Rates of Hepatitis E Virus Infection and Disease among Adolescents and Adults in Kathmandu, Nepal

Edward T. Clayson,* Mrigendra P. Shrestha, David W. Vaughn, Rapin Snitbhan, Khagendra B. Shrestha, Charles F. Longer, and Bruce L. Innis

To determine hepatitis E virus (HEV) infection and disease rates in the Kathmandu Valley of Nepal, serum was collected from 757 healthy Nepalese (ages 12–48 years) during March and September 1992 and September 1993. At each visit, reports of interval illness were obtained. Sera were examined for IgG to HEV, using a commercially available kit. Serocconversion was used as a marker for HEV infection, and an episode of hepatitis E was defined as a history of jaundice with seroconversion. Seroprevalence ranged from 16% to 31% and increased with age, whereas both infection and disease rates decreased with age. Infection and disease rates were as high as 99/1000 and 45/1000 person-years, respectively. These results highlight the importance of sporadic hepatitis E as a public health problem among adolescents and young adults in this region.

Hepatitis E virus (HEV) is believed to be the primary agent responsible for enterically transmitted non-A, non-B hepatitis. The most commonly reported signs and symptoms of this disease include malaise, jaundice, dark urine, nausea, abdominal pain, fever, hepatomegaly, and vomiting [1]. The mortality rate for hepatitis E is higher than for hepatitis A and ranges from 1%–3% among men and nonpregnant women to 12%–42% among pregnant women [2]. High rates of perinatal death are also associated with this disease [3, 4]. Hepatitis E is an important health concern in nearly all under-developed regions of the world. No vaccines for hepatitis E have been developed, and prophylaxis with immune serum globulin appears to have little or no protective effect [2].

Studies to characterize the prevalence of IgG to HEV began as a result of the recent development of serologic assays for HEV. Generally, the prevalence of antibody against HEV is low (0%–3%) in areas where no outbreaks have been reported and sporadic cases are rare (e.g., in the United States, Europe, Australia, Japan, New Zealand, South Africa, and Thailand) [5–8]. By contrast, the prevalence of antibody against HEV is higher (10%–40%) in regions where outbreaks have been reported and sporadic transmission occurs regularly, such as India, Nepal, and Tajikistan [6, 7, 9, 10]. Age-specific antibody prevalence has been examined in India and Nepal [9, 10]. In both studies, antibody prevalence was found to increase with age until about age 30 years. No increase in antibody prevalence was apparent after age 30. No sex-dependent differences in antibody prevalence were observed; on the other hand, in older age groups, the numbers of women were too small to make meaningful comparisons [10].

Little information is available for estimating rates of infection and disease in any region; existing information was determined during outbreaks. Estimates of infection and disease rates are needed to assess the public health impact of hepatitis E under conditions of sporadic transmission. We report estimates for prevalence of antibody to HEV, infection rate, case rate, and case-to-infection ratio under conditions of sporadic HEV transmission among 757 individuals in the Kathmandu Valley of Nepal.

Materials and Methods

Study area. The Kathmandu Valley has a population of approximately 1.1 million people in an area of ~900 km². The major-
Subjects. We previously reported the prevalence of IgG to HEV among 4486 healthy men, women, and children in the Kathmandu Valley [10]. The present study, an extension of the earlier work, is limited to 591 army soldiers and 166 police employees. Subjects were 12–48 years old (table 1). Age distributions among the army and police were similar except that no army subjects were <18 years of age. Seven of the police employees were female; all other subjects were male. All worked in urban areas and had access to both treated and untreated water. Serum specimens and medical histories were obtained from each subject during March and September 1992 and September 1993. Medical histories taken at each venipuncture included biographic information, history of jaundice and other illnesses, and duration of residence in Kathmandu.

Specimen handling. Blood was collected into 7-mL Vacutainer serum separator tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ), and the blood was allowed to clot for 2 h at ambient temperature. Serum was separated from clotted blood by low-speed centrifugation for 15 min. Sera were transferred into fresh tubes, frozen on dry ice, shipped to the laboratory in Bangkok, stored at −20°C, and used for determination of antibody to HEV.

Serologic assays. Sera were examined for IgG to HEV, using a commercially available ELISA (Diagnostic Biotechnology, Singapore). This test employs three recombinant HEV fusion proteins derived from expression of strongly antigenic portions of the putative structural proteins of both HEV Burma B and Mexico, 1986 isolates [11]. Assays were performed and analyzed as recommended by the manufacturer and as previously reported [1]. When done in this manner, the assay is reported to be highly specific (specificity score = 0.96) and moderately sensitive (sensitivity score = 0.82) for detecting antibody responses elicited by acute infection [1]. The sensitivity of the test to antibodies years after infection is unclear. The first, second, and third serum specimens from each person were tested together on the same plate. When any of the 3 specimens from an individual tested positive, all 3 specimens were retested. If results of first and second tests were discrepant, then specimens were tested again. To be considered positive, a specimen must have scored positive on either two of two tests or two of three tests.

Data analysis. Serologic data and medical histories obtained by structured interview at venipuncture were used to calculate initial antibody prevalence, infection rate, case rate, and case-to-infection ratio. Initial antibody prevalence was defined as the percentage of individuals with IgG to HEV at the time of the first venipuncture. The infection rate was calculated as the seroconversion rate (i.e., the proportion of susceptible persons who gained antibody responses elicited by acute infection [1]. The sensitivity of the test to antibodies years after infection is unclear. The first, second, and third serum specimens from each person were tested together on the same plate. When any of the 3 specimens from an individual tested positive, all 3 specimens were retested for confirmation. If the results of the first and second tests were discrepant, then the specimens were tested a third time. Therefore, to be considered positive, a specimen must have scored positive on either two of two tests or two of three tests.

Results

Of the 757 subjects, 186 were positive for IgG to HEV on all 3 serum specimen tests. These individuals were assumed to have been infected with HEV prior to the onset of this study. Initial antibody prevalence appeared to increase with age, rising from 16% (10/64) in the 12- to 19-year age group to 24% (114/477) in the 20- to 29-year age group to 28% (54/190) in the 30- to 39-year age group to 31% (8/26) in the 40-
to 48-year age group. Of the 186 persons with IgG to HEV, 90 reported having had jaundice before the first specimen collection, while 96 reported no history of jaundice.

During the 6 months between March and September 1992, 19 persons acquired IgG to HEV. These people also tested positive for IgG to HEV in specimens collected 12 months later in September 1993, confirming the seroconversion. Seroconversion appeared to be age-related: 17 (89%) of 19 persons were <30 years of age; none were >34. Four persons who had seroconverted to HEV also reported having jaundice during the interval. One other person reported having jaundice; however, this person had not seroconverted and was not considered to have had hepatitis E.

During the 12 months between September 1992 and September 1993, another 35 people acquired IgG to HEV. Again, seroconversion appeared to be related to age. Of the 35 people who acquired IgG to HEV during the interval, 31 (89%) were <30 years of age, and none were >37. Fourteen persons who seroconverted also reported having jaundice during the interval. Seven others also reported having jaundice; however, these people did not seroconvert and were not considered to have had hepatitis E. Overall, for the entire 18-month period, 54 people acquired IgG to HEV; 18 of them reported having jaundice during the interval (table 1).

Infection rates, case rates, and case:infection ratios were very similar among the 6- and 12-month periods (data not shown); therefore, the data for these two periods were pooled and rates were calculated for the entire 18-months (table 1). The overall infection rate was 64/1000 person-years; however, the infection rate decreased with age. The highest infection rate (99/1000 person-years) was in persons <20 years old, and this rate decreased with each successively older age group. The overall case rate was 20/1000 person-years; however, the case rate also decreased with age. The highest case rate (45/1000 person-years) was in persons <20, and this rate decreased with each successively older age group. Similarly, the highest case:infection ratio was 45 cases of jaundice per 100 infections in persons <20, and this ratio decreased with each successively older age group.

Discussion

This study extends previous knowledge of the epidemiology of HEV in the Kathmandu Valley by providing estimates of the rates of infection and disease under conditions of sporadic transmission. Our estimates of prevalence of IgG to HEV are consistent with previous reports from Kathmandu and elsewhere on the Indian subcontinent [6, 7, 9, 10]. Rates of infection and disease varied with age. The highest rates were in the 12- to 19-year-old age group, and these rates decreased with each successively older age group. No infections or cases of jaundice were observed in the oldest age group (40–48 years); however, the small number of susceptible persons >40 years old could have resulted in under-detection of cases of infection and jaundice. Nevertheless, hepatitis E has frequently been described as a disease affecting primarily adolescents and young adults, and our results are consistent with this description. The infection rates reported in this cohort confirm the high infection rates inferred from recent age-specific antibody prevalence data compiled for this region [10]. Likewise, the high disease rates among adolescents and young adults confirm the public health significance of HEV in this region.

Depending on the age group examined, the infection rate appeared to be ~2–4 times higher than the disease rate, suggesting that subclinical or anicteric HEV infection occurs more frequently than HEV infection with overt jaundice. This finding may have implications concerning the transmission of HEV. Persons in HEV-endemic areas appear to become infected and may shed HEV without recognizable illness, contributing to transmission of HEV even when few cases of jaundice are noted. In HEV-endemic areas, inapparent or anicteric infection may sustain HEV transmission between epidemics.

Even though rates of infection and disease might seem high, they may be considered minimal estimates for several reasons. Infection rates were determined on the basis of our ability to detect seroconversion using ELISA. We previously reported that this assay, although very specific (specificity score = 0.96), fails to detect infection in ~20% of patients with HEV viremia detectable with the polymerase chain reaction [1]. In addition, reinfec-
tion in persons with IgG to HEV at study entry would have gone undetected by our methods. Therefore, actual infection rates are likely higher than estimated. Similarly, our estimates of disease rates were based on a structured interview conducted after a 6- or 12-month interval. Therefore, the estimate accuracy is limited to the ability of volunteers to recall past episodes of jaundice. Failure to recall illness would lead to low disease estimates.

Care should be taken when applying our estimates of infection and disease rates to the entire population of Kathmandu. The cohort of soldiers and police consisted primarily of males; females were under-represented, and young children were not included. In addition, persons >40 years of age were also under-repre-
sented. To confirm and extend our results, similar studies should be done in additional cohorts in the Kathmandu Valley.

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References

The Impact of Active Herpes Simplex Virus Infection on Human Immunodeficiency Virus Load

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The effect of a concurrent herpes simplex virus (HSV) infection on human immunodeficiency virus type 1 (HIV-1) load was evaluated. Sixteen subjects were identified with an active HSV infection and had pre-outbreak, acute-phase, and post-outbreak plasma (n = 16) and peripheral blood mononuclear cell (PBMC) (n = 8) samples for evaluation. All subjects were treated for an acute HSV outbreak with acyclovir for 10 days, followed by chronic prophylaxis. HIV-1 plasma RNA levels were determined by branched DNA, and intracellular HIV gag mRNA copy numbers were determined by quantitative reverse transcriptase-polymerase chain reaction ELISA. Plasma virus load increased a median of 3.4-fold during the acute outbreak (range, 0- to 10-fold; P = .002), while post-outbreak levels (30- to 45 days after the appearance of lesions) remained above pre-outbreak, baseline levels in some subjects. Intracellular HIV gag mRNA increased during the outbreak as well. Thus, an acute HSV episode can result in increased HIV transcription and plasma virus load.

Human immunodeficiency virus (HIV) infection results in destruction of the immune system, leading to profound immunosuppression and the development of opportunistic infections. It is unclear whether opportunistic infections contribute directly to HIV clinical progression through T cell activation with subsequent HIV replication or through T cell destruction (or both). Reactive herpes simplex virus (HSV) infections are found with increased frequency in HIV-infected patients. In vitro and possibly in vivo studies suggest that HSV-1 can activate latent HIV or enhance replication [1-4]. A direct correlation between HSV infection and increased HIV burden in patients has not been described.

Recently, plasma HIV load measurement was found to be important in predicting disease progression or response to antiretroviral therapy [5, 6]. In addition, opportunistic infections, bacterial pneumonia including tuberculosis, interleukin-2 immunotherapy, and immunizations have all been associated with increased HIV load [7-12]. In the current study, we wished to determine whether HIV load changed significantly in subjects with active HSV infection.

Methods

Sample collection and subject selection. Subjects for this study were recruited between 1991 and 1994 for an ongoing project to investigate potential cofactors associated with HIV load variability. All subjects were veterans receiving primary HIV care at the Department of Veterans Affairs Palo Alto Health Care System. Whole blood was collected in acid citrate dextrose tubes and processed within 2 h of phlebotomy. Peripheral blood mononuclear cells (PBMC) were separated from whole blood by density centrifugation using ficoll-hypaque, washed twice with PBS, and stored as
dry pellets at $-70^\circ\text{C}$. Plasma from these samples was removed, clarified (10 min at 1000 g, room temperature), and stored at $-70^\circ\text{C}$.

Subjects experiencing an acute HSV infection or recurrent outbreak were identified retrospectively by an analysis of acyclovir prescriptions, HSV culture positivity, and chart reviews. Subjects were included if they were clinically stable with respect to HIV disease (e.g., absence of opportunistic infection or significant constitutional symptoms) for 3 months before and 3 months after the acute HSV event. Those subjects determined to be noncompliant with their antiretroviral therapy, acute acyclovir treatment, and subsequent prophylaxis during the study period (as determined by prescription audit) were excluded. No subject received any vaccinations within 3 months before the acute HSV event. All subjects with PBMC and plasma samples obtained 30–45 days before and during the acute HSV event (defined by vesicular or ulcerative disease) and 30–45 days following acyclovir therapy and continued prophylaxis were included for assessment of their HIV load.

**HIV-1 RNA quantification.** Plasma HIV-1 RNA was measured by the branched DNA (bDNA) assay according to the manufacturer’s instructions (Quantiplex 1.0; Chiron, Emeryville, CA). Cellular RNA was isolated in sequential steps by use of proteinase K digestion, phenol-chloroform extraction, isopropanol precipitation, and finally DNase treatment. Cellular RNA was quantified by spectrophotometry, and 500 ng/sample was used in a reverse transcriptase–polymerase chain reaction (RT-PCR) reaction. HIV-1 gag gene PCR product (SK38 and SK39) was quantitated with an HIV detection kit (Synthetic Genetics, San Diego) and a previously described external RNA standard [13]. HIV-1 mRNA copy number was expressed as $\log_{10}$ copies per microgram of cellular RNA. Immune complex–dissociated (ICD) p24 antigen concentrations in plasma were determined by a commercially available kit (Coulter, Hialeah, FL). CD3/CD4 lymphocyte counts and percentages were determined by a reference laboratory (Pathlabs, San Jose, CA). Analyses for changes in bDNA, ICD p24 antigen levels, and CD4 lymphocyte counts were made by use of the Wilcoxon matched pairs test. Significance was defined as $P < .05$.

**Results**

Thirty-six subjects were identified as having probable or confirmed HSV infection. Sixteen male subjects were identified who had experienced an active infection with cutaneous or mucocutaneous HSV infection and for whom plasma or PBMC samples were available for all three time points. Clinical characteristics of the subjects are provided in table 1. None of the subjects were receiving chronic acyclovir prophylaxis on entry to the study. Eight subjects had received continuous antiretroviral therapy (2 zidovudine, 2 didanosine, 4 combination) for at least 3 months before and throughout this study. Five subjects had positive HSV cultures from lesions. Eleven subjects were presumed to have HSV infections on the basis of a clinical presentation of vesicular or ulcerative lesions and a previous episode with a positive HSV culture. One subject was HSV culture–negative but Tszank smear–positive. Subtype of HSV was not reported by the reference laboratory at the time. At the acute presentation, all subjects received oral acyclovir (200 mg orally 5 times daily for 10 days), followed by 400 mg twice a day for chronic prophylaxis. Lesions resolved clinically in all study subjects. Mean CD4 lymphocyte counts for the 11 subjects for whom counts were obtained prior to and following the HSV outbreak were $84 \pm 27$ and $105 \pm 35$ cells/mm$^3$, respectively ($P > .05$). CD4 cell counts during the acute episode were available for only 2 subjects. A fall in both absolute CD4 lymphocyte count and percentage was seen in these 2 subjects at the time of acute HSV diagnosis. This was followed by a return to baseline within 3 months. Only 5 subjects had detectable ICD p24 antigen levels at baseline. No change in ICD p24 antigen levels over time was seen in these 5 evaluable subjects (data not shown). None of the remaining 11 subjects had detectable ICD p24 levels at any of the time points during the study.

Fourteen of 16 subjects demonstrated an increase in plasma HIV-1 RNA during the acute HSV episode (figure 1A, B). Median HIV plasma RNA copy numbers before, during, and after the HSV outbreak for the three collection time points were 73, 250, and 94 copies ($\times 1000$)/mL of plasma, respectively. Plasma HIV RNA levels at baseline and in the period following the acute HSV event were significantly lower than during the acute episode ($P = .002$ and $P = .001$, respectively). The post-outbreak plasma HIV-1 RNA level was greater than that at baseline in 10 of 16 subjects ($P = .14$). The effect of antiretroviral therapy on virus load was also analyzed. Baseline median virus load was not significantly different between subjects receiving antiretroviral therapy and subjects who were not (55,000 vs. 80,000/mL; $P = .16$). However, the median increase during the acute episode was significantly greater in those subjects who were not receiving antiretroviral therapy (512,000 vs. 146,000/mL; $P = .03$). Post-episode median virus load remained higher in those subjects not receiving antiretroviral therapy than in those who were (121,000 vs. 53,500/mL; $P = .02$).

PBMC samples for all three time points were available for only 8 subjects. PBMC mRNA was detectable and quantifiable in 4 of 8 subjects before the HSV outbreak, in 8 of 8 during the acute episode, and in 7 of 8 following the acute episode (figure 1C). Thus, 3 subjects changed from having undetectable to detectable levels in their PBMC during the outbreak. The 4 subjects with measurable cellular mRNA at all three collection points had median mRNA levels of 299, 837, and 396 copies of HIV-1 gag mRNA/µg of PBMC RNA in baseline, acute, and post-outbreak periods, respectively.

**Discussion**

The data presented here demonstrate that HIV RNA levels can increase during active HSV infection. The level of increase is greater than one would expect as a result of biologic or assay variability [14]. We found that while this increase was transient in conjunction with the clinical resolution of the herpetic le-
Table 1. Clinical and laboratory characteristics of patients with acute HSV infection.

<table>
<thead>
<tr>
<th>Treatment, subject</th>
<th>CD4 cell count/mm³</th>
<th>Diagnosis</th>
<th>Baseline</th>
<th>Acute</th>
<th>Post</th>
<th>Antiretroviral</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antiretroviral treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>Cx</td>
<td>116,000</td>
<td>302,000</td>
<td>87,000</td>
<td>ddI</td>
</tr>
<tr>
<td>13</td>
<td>120</td>
<td>P</td>
<td>12,600</td>
<td>14,500</td>
<td>13,000</td>
<td>ddI</td>
</tr>
<tr>
<td>10</td>
<td>176</td>
<td>P</td>
<td>104,000</td>
<td>235,000</td>
<td>93,000</td>
<td>ZDV</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>P</td>
<td>12,000</td>
<td>30,000</td>
<td>20,000</td>
<td>ZDV</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>P</td>
<td>39,000</td>
<td>117,000</td>
<td>46,000</td>
<td>ZDV/ddI</td>
</tr>
<tr>
<td>5</td>
<td>323</td>
<td>Cx</td>
<td>69,000</td>
<td>170,000</td>
<td>61,000</td>
<td>ZDV/ddI</td>
</tr>
<tr>
<td>7</td>
<td>66</td>
<td>P</td>
<td>41,000</td>
<td>185,000</td>
<td>101,000</td>
<td>ZDV/ddI</td>
</tr>
<tr>
<td>15</td>
<td>336</td>
<td>P</td>
<td>122,000</td>
<td>122,000</td>
<td>46,000</td>
<td>ZDV/ddI</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>114</td>
<td>146,938</td>
<td>58,375</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td>119</td>
<td>42,118</td>
<td>30,984</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
<td>93</td>
<td>55,000</td>
<td>53,500</td>
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</tr>
<tr>
<td><strong>No antiretroviral treatment</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>P</td>
<td>683,000</td>
<td>7,832,000</td>
<td>1,973,000</td>
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</tr>
<tr>
<td>2</td>
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<td>382,000</td>
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<tr>
<td>6</td>
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</tr>
<tr>
<td>9</td>
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</tr>
<tr>
<td>11</td>
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<td>795,000</td>
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</tr>
<tr>
<td>12</td>
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<td>T</td>
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</tr>
<tr>
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<td>9</td>
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<td></td>
</tr>
<tr>
<td>16</td>
<td>14</td>
<td>P</td>
<td>36,000</td>
<td>266,000</td>
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<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>52</td>
<td>158,875</td>
<td>361,250</td>
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</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td>106</td>
<td>217,008</td>
<td>652,616</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
<td>12</td>
<td>80,500</td>
<td>121,000</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td>Mean</td>
<td>111,663</td>
<td>760,656</td>
<td>209,813</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SD</td>
<td>159,060</td>
<td>1,899,837</td>
<td>473,002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Median</td>
<td>73,000</td>
<td>250,500</td>
<td>93,500</td>
</tr>
</tbody>
</table>

**NOTE.** Cx, culture; P, presumed; T, Tszank smear.

The magnitude of virus load response may be related to the size, extent, or length of time that HSV lesions are present or to concomitant antitherpetic or antiretroviral therapy. Schaker et al. [15] found a smaller median increase (51% over baseline) in HIV-1 plasma RNA in the days surrounding an acute HSV-2 event. Semple et al. [16] reported on 1 subject with focal disease who had a 10-fold transient increase in virus load during an HSV infection. No details regarding acyclovir therapy were provided. We found greater HIV load increases in those subjects who were not receiving antiretroviral therapy. Whether subjects with undetectable virus load as a result of effective antiretroviral therapy still demonstrate these increases remains to be studied.

We have demonstrated that HIV load first increases and then decreases in the weeks surrounding an acute HSV episode in the presence of acyclovir therapy and subsequent prophylaxis. Since this increase appears to be short-term, the overall impact on the individual patient may not be great. Similar short-term increases in HIV plasma RNA levels have been reported for HIV-seropositive patients with other coinfections [7–9], patients receiving immunomodulators such as interleukin-2 [10], and patients receiving immunizations with tetanus toxoid and pneumococcal vaccine [11, 12]. Serial samples collected after immunization with tetanus toxoid or pneumococcal vaccination suggest that virus load elevations of 2- to 100-fold are transient in nature and may be related to HIV status (CD4 cell count, degree of immunosuppression) or antibody response. The percentage of activated T cells was also increased. Bush et al. [7] found that in 13 subjects with bacterial pneumonia, plasma virus load increased by a mean of 0.5 log10 (3-fold) compared to pre-pneumonia levels. Donovan et al. [8] found that in a small series of patients with active opportunistic infections, virus load transiently increased by an average of 7-fold (0.85
Figure 1. A. Median plasma HIV RNA copy no. per milliliter from 16 study subjects before (pre), during (acute), and after (post) herpetic outbreak. Line connects median values, large box encompasses 75th percentile, and bars indicate minimum and maximum values. B. Plasma HIV-1 RNA copy nos. for individual subjects during acute HSV infection. Values inside graph and bold line indicate means. C. Peripheral blood mononuclear cell HIV-1 gag mRNA log copy no. per µg of cellular RNA from 8 subjects before, during, and after herpetic outbreak. Horizontal line indicates threshold of assay detection.
log₁₀). Four of 15 subjects had virus loads that remained ≥2-fold greater than baseline levels after resolution or therapy. In their study, as in ours, no significant change in ICD p24 antigen levels was seen. Michael et al. [9] described a 5-fold-greater level of virus load in subjects with active pulmonary tuberculosis compared with that in controls. However, subjects with tuberculosis, after receiving 6 months of antituberculosis therapy, had a 70-fold-greater virus load. Another study found that patients with latent tuberculosis (positive tuberculin skin testing) did not have significantly different virus loads than did those with negative skin test reactions [17].

Although the number of subjects in our study was small, a trend toward higher plasma and intracellular HIV RNA levels was seen despite resolution of HSV lesions and chronic acyclovir prophylaxis. This may represent sampling error due to the timing of post-exacerbation samples. Alternatively, since the optimal acyclovir HSV prophylaxis dose in HIV disease is not known, it may represent an inadequate subclinical effect. Whether these changes in HIV load will affect disease progression will likely depend on the extent of concomitant disease, the length of time HIV load is elevated to high levels (e.g., >10⁶ copies/mL of plasma), the antiretroviral regimen, and the current immune status of the individual patient. In any event, when considering the use of virus load testing to monitor patients, active HSV and other infections must be considered in the interpretation of the results.

Whether concomitant HSV infection is a significant cofactor in HIV progression remains to be determined. Studies addressing whether chronic acyclovir prophylaxis decreases HIV progression have yielded conflicting results. Virologic monitoring was not performed in these HSV prophylaxis studies to determine whether increases in virus load affected morbidity and mortality. If antigenic stimulation from immunization or concomitant infection results in latently infected T cell activation in vivo, then suppression of these events may be clinically beneficial. Further studies are necessary to determine whether active HSV infection and/or antiviral intervention against HSV affects the pathogenesis of HIV disease.

Acknowledgment

We thank the patients of the HIV Clinic at the Veterans Affairs Palo Alto Health Care System for their participation in a longitudinal study of potential cofactors in HIV disease.

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12. Brichacek B, Swindells S, Janoff EN, Pirruccello S, Stevenson M. In-progression have yielded conflicting results. Virologic monitoring was not performed in these HSV prophylaxis studies to determine whether increases in virus load affected morbidity and mortality. If antigenic stimulation from immunization or concomitant infection results in latently infected T cell activation in vivo, then suppression of these events may be clinically beneficial. Further studies are necessary to determine whether active HSV infection and/or antiviral intervention against HSV affects the pathogenesis of HIV disease.


Human Herpesvirus 8 Infection Occurs following Adolescence in the United States

Andrew Blauvelt, Shizuko Sei, Pamela M. Cook, Thomas F. Schulz, and Kuan-Teh Jeang

Most recent evidence suggests that human herpesvirus 8 (HHV-8) infection is restricted to persons with Kaposi’s sarcoma (KS) or to persons who may subsequently develop KS. To accurately determine the prevalence of infection in the United States, children and adults with AIDS were examined for evidence of HHV-8 infection to see whether HHV-8 (like other herpesviruses) would be readily detected in immunosuppressed persons. By use of nested polymerase chain reaction, DNA specific for HHV-8, Epstein-Barr virus, and cytomegalovirus was detected in blood leukocytes from 0, 26 (51%), and 9 (18%), respectively, of 51 children. Similarly, HHV-8–specific antibodies were not detected in analyses of sera from the children. By contrast, HHV-8 DNA was detected in 9 (27%) of 33 adult AIDS patients without KS. These findings suggest that the pattern of transmission of HHV-8 in the United States differs from that of other herpesviruses in that primary infection occurs predominantly in adults.

Human herpesvirus 8 (HHV-8), also known as Kaposi’s sarcoma (KS)–associated herpesvirus, is a newly described γ-herpesvirus with sequence homology to Epstein-Barr virus (EBV) [1]. Through both DNA and serologic assays, HHV-8 is associated with the diseases KS [1–6], body cavity–based lymphoma (BCBL) [7], and Castleman’s disease [8]. However, the prevalence of HHV-8 infection in the general population and, specifically, the mode and age of primary infection remain controversial. DNA analyses of semen from human immunodeficiency virus (HIV)–negative heterosexual men from the United States and Italy suggest that HHV-8 infection is widely prevalent (23% and 91%, respectively) in general populations [9, 10]. Serologic assays in healthy adult blood donors, however, have yielded conflicting results, with HHV-8–specific antibodies detected in <5% [3–5] and up to 25% [6] of persons tested. In immunocompetent children, seroprevalence has been reported to be 0 [5] and <4% [6]. Because herpesvirus infections are often reactivated with immunosuppression [11, 12], we hypothesized that HHV-8 might be more readily detected in an immunosuppressed population of children. Thus, we examined US children with AIDS for HHV-8 infection. For comparisons, we also tested the same children for presence of EBV- and cytomegalovirus (CMV)-specific DNA in peripheral blood mononuclear cells (PBMC). EBV and CMV are herpesviruses known to be widely prevalent and transmitted early in life. In addition, US adults with AIDS and without the diagnosis of KS (i.e., similar to the children in the degree of immunosuppression) were examined for evidence of HHV-8 DNA in PBMC. By comparing the prevalence of HHV-8 infection in immunosuppressed US children and adults, the aim of these analyses was to determine an accurate prevalence of HHV-8 infection in the general US population.

Materials and Methods

Patients. Fifty-one HIV-infected children with AIDS by Centers for Disease Control and Prevention criteria [13], who were enrolled in clinical trials at the HIV and AIDS Malignancy Branch (formerly part of the Pediatrics Branch), National Cancer Institute, were studied. Thirty-three adults with AIDS [14] and without KS, who were enrolled in clinical trials at the Laboratory of Immunoregulation, National Institute for Allergy and Infectious Diseases, were also studied. Demographic and clinical information for all of the patients was collected through chart review.

Detection of virus-specific DNA by polymerase chain reaction (PCR). PBMC were isolated by density gradient centrifugation of heparinized blood. Total DNA was isolated after overnight digestion at 55°C in 4 mL of solution containing 10 mM TRIS-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% SDS, and 400 μg/mL protease K, followed by phenol-chloroform extraction and ethanol precipitation. The concentration of DNA was determined by optical density readings at a wavelength of 260 nm by use of a spectrophotometer. For positive viral DNA controls, DNA was
isolated from the HHV-8+EBV+ BCBL cell line BC-1 (gift of Ethel Cesaran, New York Hospital-Cornell Medical Center, New York) [15], the HHV-8+EBV+ BCBL cell line BCBL-1 (NIH AIDS Research and Reference Reagent Program, Rockville, MD), or EBV-specific and CMV-specific DNA (purchased from Advanced Biotechnologies, Columbia, MD).

Oligonucleotide primers (Genosys Biotechnologies, The Woodlands, TX) used in PCR assays were as follows (5’–3’): HHV-8 ORF26 (outer sense), AGCTAGCATGTCTACCCCCCA; HHV-8 ORF26 (outer antisense), ATCGTCAAGCCTCGCCAGGG; HHV-8 ORF26 (inner sense), GAAAGGATCCCCACATGTG; HHV-8 ORF26 (inner antisense), ATCCGGTTGTCATC- GTCCA; EBV EBNA-1 (outer sense), CCAAGACCTGGACC- CGGG; EBV EBNA-1 (outer antisense), CAAAGGGAGAC- GACTCAAT; EBV EBNA-1 (inner sense), TGGAGGCGGCG- CAAAAAG; EBV EBNA-1 (inner antisense), GACATTGG- GAATAGCAAGG; CMV IE1 (outer sense), TTTCGAAGTCCT- CACCCCAT; CMV IE1 (outer antisense), GCAACCTGGAC- CATTGCC; CMV IE1 (inner sense), GCAGTTCCATGGCCACCTG; β-globin (outer sense), TCTGCGTGTACTGCC- CTGTG; β-globin (outer antisense), CCTTACCCTTAGGTT- GACCC; β-globin (inner sense), AACAGTGATGAAGTT- GCTTC; β-globin (inner antisense), CAGGAGTGAGTAAAT- GGTGG; β-globin (inner antisense), CAGGAGTGGACAGAT- CCCCC.

All of the following PCR reagents were purchased from Perkin-Elmer Cetus (Foster City, CA). Each PCR reaction was carried out in thin-walled tubes in a volume of 100 μL with use of DNA-free water as diluent. Each tube contained 1× PCR buffer, 1.5 mM MgCl2, 200 μM each dNTP, 2.5 U of AmpliTaq Gold, 0.5 μM each sense and antisense outer primer, and 1 μg of target DNA. Negative controls contained all reagents except DNA template. These negative tubes flanked positive controls during the setup of each experiment. Samples were denatured for 2 min at 95°C, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and ending with a single incubation for 5 min at 72°C with use of a thermal cycler. For nested PCR, DNA template consisted of 5 μL of outer PCR product mixed with corresponding inner primer sets; otherwise, PCR reactions were carried out as described above. PCR product (20 μL) was electrophoresed and visualized in ethidium bromide−stained 1% agarose gels.

To guard against contamination, extraction of DNA, setup of outer and inner PCR, and electrophoresis were performed in separate locations with separate sets of equipment. Barrier-resistant pipet tips were used throughout, and gloves were changed at each step. To determine the sensitivity of our PCR, serial dilutions of BC-1 DNA were mixed with 1 μg of DNA obtained from the transformed human embryonal kidney cell line 293 (American Type Culture Collection, Rockville, MD). BC-1 cells were previously shown to contain 40–80 copies of HHV-8/cell [15].

Detection of HHV-8–specific antibodies directed against latent and lytic antigens. For the 51 children, clotted blood samples were centrifuged, and sera were collected and stored at −20°C until assayed for HHV-8–specific antibodies. Antibodies directed against latently expressed HHV-8 antigens were assessed by an immunofluorescence assay, whereas antibodies directed against a lytically expressed antigen (i.e., a truncated recombinant minor capsid protein encoded by ORF65) were tested by an ELISA on two separate occasions, both as described in detail [5]. Briefly, for the immunofluorescence assay, sera at a 1:200 dilution (previously determined to represent the best dilution for avoiding nonspecific staining and for detecting specific staining) were incubated with BC-1 cells, which are BCBL-derived cells latently infected with HHV-8, subsequently incubated with fluorescein isothiocyanate–conjugated rabbit anti-human IgG (Dako, Carpinteria, CA) after a series of washes, and examined by an immunofluorescence microscope. For the ELISA, sera were tested at 1:100 dilution, and the cutoff value to define reactive sera was selected as SSD above a mean value derived from previously tested nonreactive sera. Sera from KS patients and from healthy blood donors (previously determined to be HHV-8 antibody−positive and antibody−negative, respectively, by both assays) were used as controls when testing the children’s sera for this study. If results from these experiments were indeterminate, Western blots were performed for further clarification, also as described in detail [5].

Results

Demographic and clinical information for the children and adults is outlined in table 1. The median age for the children was 7 years (range, 0.5–18); 10 children were between the ages of 13 and 18 years, 12 between the ages of 8 and 12 years, 16 between the ages of 3 and 7 years, and 13 under the age of 3 years. For the adults, median age was 37 years (range, 26–55). The median CD4 cell count for the children and adults was 185/mm3 (range, 2–2140) and 119/mm3 (range, 4–200), respectively. Interestingly, 19 children had biopsy-proven lymphoma or lymphoproliferative disease, although none had BCBL, the type of lymphoma previously linked with HHV-8 [7]. Of note, none of the children studied reported prior sexual
Discussion

We found that US children with marked immunosuppression showed no evidence of HHV-8 infection as determined by use of all currently accepted techniques for detecting this virus. These data are consistent with HHV-8 seroprevalence studies previously reported for healthy children [5, 6] and are in contrast to transmission patterns reported for most other herpesviruses (e.g., infection with the prototypic γ-herpesvirus EBV and the prototypic β-herpesvirus CMV had already occurred in at least 51% and 18%, respectively, of our childhood patient population). Of note, we recognize that the prevalence of HHV-8 infection in children from east and central Africa may show a different pattern compared with that in US children, since KS occurs not infrequently in young central Africans, and HHV-8 seroprevalence in healthy Ugandan adults has been reported to be 50%–60% [4, 5].

To our knowledge, this is the first comparative detection study of HHV-8 DNA in the blood of children and adults with AIDS. Although we compared different age groups with similar levels of immunosuppression, we acknowledge that these groups also differed in their sexual activity. To prove that HHV-8 is a sexually transmitted virus, as suggested by our study and many others, it will be important to compare HHV-8 prevalence in sexually active adolescents and young adults with that in their sexually inactive counterparts.

Our results are derived from a population of immunosuppressed children. Considering that most herpesvirus infections are reactivated and more readily detectable during times of immune compromise [11, 12], we believe that our pediatric AIDS cohort gives an accurate measurement of the prevalence of HHV-8 in US children. We are aware that there may be unidentified demographic differences between children with AIDS and healthy children and that those factors might preclude direct comparisons of these populations. However, HIV infection and immunosuppression appear to be the only two differences when our children are compared with their healthy counterparts. Thus, our findings suggest that primary HHV-8 infection occurs following adolescence in the United States.

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References


Absence of Detectable Human Herpesvirus 8 in the Semen of Human Immunodeficiency Virus–Infected Men without Kaposi’s Sarcoma

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The prevalence of human herpesvirus 8 (HHV-8)/Kaposi’s sarcoma (KS)–associated herpesvirus was investigated in the semen of 99 human immunodeficiency virus (HIV)–infected men (median CD4 cell count, 357/mm³) by use of a polymerase chain reaction (PCR) assay capable of detecting <10 copies of HHV-8 DNA. Of the subjects, 95 (96%) self-identified as men who have sex with men (MSM), and 3 had a history of clinical KS. Seminal cell specimens were negative for HHV-8 in 98 subjects. None of the 26 without KS (27.1% of 96 tested) who were seropositive for HHV-8 by IFA for latency-associated nuclear antigens had HHV-8 detected in their semen. The only subject with any evidence for seminal HHV-8 DNA was seropositive for HHV-8 and had active KS. HHV-8 was detected in 10 (10.4%) of 96 peripheral blood mononuclear cell specimens. The prevalence of HHV-8 DNA by PCR in semen of HIV-infected MSM without KS is low.

Kaposi’s sarcoma (KS) was recognized as an AIDS-associated malignancy among men who have sex with men (MSM) at the onset of the AIDS epidemic. In 1994, Moore and Chang [1] detected DNA sequences of a herpesvirus now known as human herpesvirus 8 (HHV-8) in KS lesions from men with and without human immunodeficiency virus (HIV) infection. Epidemiologic evidence suggests that HIV-associated KS is related to a sexually transmitted pathogen. KS is much more frequent among HIV-infected MSM than among those infected through other routes. Given the evidence that KS is a sexually transmitted disease, one would anticipate that HHV-8 might be detected in the semen of HIV-infected men. Studies of whether HHV-8 is found in semen have had conflicting results [2–6], and laboratory contamination of polymerase chain reaction (PCR) products in HHV-8 specimens has been reported [7]. Our laboratory has a long-standing interest in sexually transmitted herpesviruses and extensive experience detecting their presence in genital secretions. As such, we sought to evaluate the prevalence of HHV-8 in the semen of HIV-infected MSM.

Methods

Subjects. The 99 semen specimens examined by PCR for HHV-8 were originally collected during a prospective longitudinal study of HIV and cytomegalovirus (CMV) shedding in semen from HIV-infected men [8]. These men were HIV-infected volunteers recruited from University of Washington–affiliated clinics and the general Seattle–King County area. At study entry, semen and blood samples were obtained. Subjects then provided 3 semen and blood samples over a 2-month period.

Median CD4 cell count of the blood samples was 357/mm³ (range, 0–1034). Median age was 36 years (range, 23–55). Of the 99 participants, 95 (96%) self-identified as MSM. One subject stated that he had been diagnosed with KS in the past but had no ongoing lesions (resolved KS), while 2 subjects had active KS and were undergoing chemotherapy during the study period. All 3 KS patients had skin involvement but no known end-organ disease. Nineteen subjects (19%) were receiving acyclovir and 2 (2%) were receiving ganciclovir or foscarnet.

Semen processing and virologic studies. Semen specimens were obtained after 2 days of abstinence and processed within 2 h. ~0.25 mL of each ejaculate was separated into seminal cell pellets and supernatants by centrifugation at 2500 g for 2 min. Peripheral blood mononuclear cells (PBMC) were isolated by use of standard ficoll-hypaque density gradient centrifugation, and 2 × 10⁶ cells from each PBMC specimen were frozen at −70°C for subsequent extraction and PCR analysis.

DNA was isolated from the seminal cell pellets, from 50–200 μL of semen supernatant (depending on the amount available), and from 2 × 10⁶ PBMC by use of standard phenol-chloroform extraction after denaturation with guanidinium. DNA was ethanol-precipitated along with a glycogen carrier and then resuspended in 200 μL of 10 mM TRIS (pH 8.0). Ten microliters was used for each PCR reaction. KS-1 and -2 primers were used to amplify the KS330 Bam43 fragment of ORF 26 of HHV-8 [1]. HHV-8–specific PCR products were detected after 35 cycles by liquid hybridization by use of the 32P-labeled oligonucleotide probe, KS-P, specific for the KS-1,2 amplimer [1]. As a positive control, a plasmid containing the KS-1,2 target DNA sequence was obtained by cloning the PCR product into the TA vector (InVitrogen, San Diego). Plasmid DNA was quantitated by optical density at 260 nm, and serial log₁₀ dilutions of plasmid DNA from
$10^4$ to $10^5$ copies were amplified with each PCR run to generate a standard curve. The sensitivity of this PCR assay was determined to be between 1 and 10 copies of HHV-8 DNA. No cross-reactivity was seen with any of the other human herpesviruses.

To detect false-negative PCR results, 50 copies of internal positive control DNA, KS-fly2, was added to each KS-1,2 reaction. KS-fly2 is identical to the KS330 Bam233 PCR product except that the probe hybridization sequence is replaced by a 21-bp Drosophila melanogaster DNA sequence [9]. Amplification was confirmed by liquid hybridization with the $^{32}$P-labeled oligonucleotide probes specific for the D. melanogaster DNA sequence. All samples were amplified with the KS-fly2 probe, indicating no inhibition of the PCR reaction.

A separate control experiment was done in which 100 µL of HHV-8–infected saliva [10], containing 2500 copies of HHV-8 as detected by the KS-1 and KS-2 primers, was added to HIV-infected fresh KS-negative donor semen. The semen supplemented with infected material had a strong HHV-8 signal, while un-supplemented semen did not. To ensure that a negative signal for HHV-8 was not due to failure to extract DNA from the semen sample, 25 random specimens were analyzed by PCR for the human β-globin gene [11], and all were positive at $>1000$ DNA copies/reaction.

All assays included aliquots of frozen cultured HSB-2 cells (a T cell line without HHV-8) as a negative control. PCR reactions without added DNA were also included with each PCR run.

All seminal cell specimens were assayed for CMV by the same technique used in the KS PCR assay. CMV 431 (CCCGGCTCC-AATGCACCGTTCC) and 559 (AGGCCGTGACGCTGGGAG-GTCT) primers were used to amplify a 150-bp fragment of a conserved region of the immediate-early gene [12, 13]. CMV-specific PCR products were detected after 30 cycles by liquid hybridization with $^{32}$P-labeled oligonucleotide probe CMV 55 (TCTATGGAGGTCAAAACAGCG) specific for the CMV 431, 559 amplimer [12, 13].

Selected specimens were also evaluated by nested PCR assays in the laboratory of one of us (T.M.R.). The assays use primers targeting either the region upstream of the KS330 fragment in the minor capsid gene or a region in the HHV-8 glycoprotein B gene (unpublished data). Nested PCR was done on all seminal cell, seminal plasma, and PBMC specimens from the participants with known KS.

Serologic testing to detect antibodies to HHV-8, using an IFA for latency-associated nuclear antigens (LANA), was performed as previously described [14].

### Results

All 96 seminal cell specimens from the 96 HIV-infected men without diagnosed KS were negative for HHV-8 by PCR by use of the KS330 Bam233 assay (table 1). Seminal plasma samples were available from 67 subjects; all were negative for HHV-8 DNA. CMV was detected by PCR in 55 (57%) of the 96 seminal cell specimens. The PBMC of 96 subjects were also tested for HHV-8 by PCR, and 10 (10.4%) had HHV-8 DNA identified. Seven of these 10 men had PBMC specimens from a second time point that also demonstrated HHV-8 by PCR. Twenty-six (27.1%) were seropositive for HHV-8 by LANA assay. There was no association between treatment with acyclovir, ganciclovir, or foscarnet and KS PBMC or serologic results ($\chi^2; P > .05$).

Of the 3 patients with known KS, 1 with active clinical KS had 1 of 3 serial seminal cell specimens positive for HHV-8; the 3 serial seminal plasma specimens were negative, as were the 3 serial PBMC specimens. The 2 other patients with known KS had no positive samples in semen or PBMC. Four seminal cell specimens, 2 seminal plasma samples, and 3 PBMC specimens were negative in 1 patient tested; the other patient had 3 seminal cell and seminal plasma specimens and 2 PBMC specimens that tested negative. All seminal cell, seminal plasma, and PBMC specimens from the 3 participants with KS were negative by nested PCR for both the glycoprotein B and upstream minor capsid genes of HHV-8. The 1 subject in whom we detected HHV-8 DNA in semen by the KS330 Bam233 assay was also seropositive for HHV-8.

### Discussion

Reports from the United States and England have been conflicting concerning the presence of HHV-8 in semen. Using nested primers, Lin et al. [2] found a high prevalence in the semen of HIV-infected homosexual men (30/33, 91%) as well as a lower prevalence in healthy HIV-negative donors (7/30, 23%). Gupta et al. [3] reported that HHV-8 is present in the semen of 14% (2/14) of HIV-infected men with KS. In contrast, Weiss [4] did not find the virus in semen from healthy donors at fertility clinics, and Ambrozik et al. [5] reported 5 subjects with clinical KS, all without detectable HHV-8 in their semen.

Italian investigators reported a large proportion of ejaculates (30/33, 91%) and prostate tissue (7/16, 44%) from healthy men as HHV-8–positive [6]. These findings could in part be due to different prevalences of HHV-8 in various geographic areas or populations. For example, classical KS occurs in elderly Mediterranean men.

It is possible that our negative results in semen with positive results in PBMC could be due to different strains in PBMC.
and semen. However, we were able to demonstrate HHV-8 in PBMC from 10.4% of the HIV-infected men in our cohort; these data are similar to those reported in other series. Another explanation could be that different populations could be infected with different strains of HHV-8. Neither of these explanations seems likely, since we are using the same primer set that has given positive results for other investigators in the United States, and the sensitivity of our assay is equal to or better than that of other investigators’ assays.

Improper handling can result in false-negative PCR results. Several lines of evidence suggest that this was not the case in our study. First, semen specimens were processed rapidly and frozen at −70°C. Also, although inhibition can cause false-negative PCR, our internal positive control DNA, KS-fly2, rules out such false-negatives. In addition, we were able to detect CMV DNA in 57% of the specimens and cellular material, as evidenced by amplification of the human β-globin gene in all tested specimens. That our assay was capable of detecting HHV-8 DNA is demonstrated by our experiment in which infected saliva was added to donor semen and subsequently successfully identified by PCR as well as by our detection of HHV-8 in PBMC.

Although HHV-8 could be present in semen in amounts below the detection limits of our assay, the sensitivity of the KS330 Bam33 assay was determined to be <10 copies of HHV-8 DNA, and the semen specimens from the 3 men with KS were also found to be negative by a nested PCR assay. The positive result obtained with the KS330 Bam33 assay on 1 of the semen samples of 1 KS patient suggests that patients with KS may have low intermittent titers of virus in semen.

Studies thus far have indicated that acyclovir does not have any protective effect against KS, while foscarin and possibly ganciclovir may [15]. While a substantial minority of subjects received acyclovir, few were taking ganciclovir or foscarin. Thus, it is unlikely that medication prevented HHV-8 shedding and caused our negative semen results.

Comparing the detection of HHV-8 DNA in PBMC with the presence of anti–HHV-8 LANA antibodies among the patients without KS, we found only a small overlapping subset of patients positive by both assays. Eight patients tested HHV-8–positive by PCR of PBMC but were negative by serology. Interestingly, 2 of the 3 patients with clinical KS were negative for HHV-8 by both PBMC PCR and serology. Seronegativity in high-risk subjects without clinical KS could represent a seroconversion window. Seronegativity in subjects with clinical KS may reflect poor antibody response in immunocompromised persons; both of the KS patients who were seronegative for HHV-8 had CD4 cell counts well under 50/mm³. It is also possible that the 2 seronegative KS patients may simply reflect the insensitivity of the LANA serologic assay coupled with the small number (3) of men in the KS sample; even in larger series of HIV-infected men with KS, only ~80% are seropositive by the LANA assay [14].

The main limitation of our study is the relatively small number of participants with known clinical KS (3/99). However, it is clear that in HIV-infected men without clinical KS, semen does not have demonstrable HHV-8 by PCR, and its role in HHV-8 transmission is uncertain. Further research regarding other potential modes of HHV-8 transmission will clarify the currently conflicting findings concerning HHV-8 in semen.

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References

Circulating Cytomegalovirus (CMV)-Infected Endothelial Cells in Marrow Transplant Patients with CMV Disease and CMV Infection

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Circulating cytomegalovirus (CMV)-infected endothelial cells (CCIC) have been found in immunocompromised patients with CMV disease and have been associated with disease severity. The frequency of CCIC in marrow transplant recipients was studied to determine its use for distinguishing between CMV pneumonia and asymptomatic CMV infection. CCIC were found in 13 (81%) of 16 patients with CMV pneumonia (5/6 without copathogen; 7/10 with copathogen) and in 10 (50%) of 20 patients with asymptomatic CMV antigenemia at different levels. There was no statistically significant association for the incidence nor the quantitative level of CCIC in patients with asymptomatic CMV antigenemia, CMV pneumonia with or without copathogen, or different levels of CMV virus load measured by antigenemia. The high incidence and quantitative level of CCIC in patients without clinical disease suggests a role for CCIC in CMV dissemination rather than its being a specific marker of organ or tissue damage after marrow transplantation.

Cytomegalovirus (CMV) disease is a common infectious complication in allogeneic marrow transplant recipients, and CMV pneumonia continues to be associated with high morbidity and mortality in this population [1]. Its diagnosis is based on clinical criteria (i.e., hypoxemia, radiographic changes) and laboratory confirmation by CMV detection in bronchoalveolar lavage (BAL) fluid or lung biopsy [2]. Since CMV can be isolated from BAL fluid in a significant number of patients without CMV disease [3], its pathogenic role is often unclear in patients with CMV isolated from BAL fluid in the presence of other significant pathogens (e.g., Aspergillus species or respiratory viruses). Current clinical practice is to interpret any detection of CMV in BAL fluid in the presence of clinical signs or symptoms as CMV pneumonia, but this leads to some degree of overdiagnosis and overtreatment.

The available parameters reflecting systemic virus load, that is, pp65 antigenemia and CMV DNA polymerase chain reaction (PCR), cannot distinguish between CMV disease and CMV infection. Although a correlation exists between disease severity and virus load measured by levels of antigenemia or DNA PCR, CMV pneumonia can still occur at low levels of virus load, and not all patients with high virus load develop CMV disease [4, 5]. Therefore, these parameters are of limited value in the distinction between CMV as the pathogen causing pneumonia and CMV as a bystander pathogen.

Circulating CMV-infected endothelial cells (CCIC) have been detected in the mononuclear cell fraction of peripheral blood in immunocompromised patients with CMV disease. In the two initial reports, the number of these cells was associated with severity of infection and presence of organ involvement [6, 7]. Several studies give insight into the possible role of CCIC in the complex pathogenesis of CMV pneumonia after marrow transplantation. Endothelial cells have been shown to be permissive for CMV replication, and infected cells exhibit the pathognomonic nuclear inclusions [7–9]. Indeed, autopsy studies of marrow transplant recipients with CMV pneumonia demonstrated CMV infection of endothelial cells in patients with CMV pneumonia [8]. Beschorner et al. [10] reported that, in disseminated CMV pneumonia, CMV infection often occurs as either necrotizing or diffuse foci. The pathologic features of CMV pneumonia with multiple necrotizing foci of cytomegalic cells suggest miliary dissemination [10]. In addition, transmission is possible between endothelial cells and monocytes bidirectionally, and enhanced expression of leukocyte adhesion molecules could further facilitate contact to leukocytes [11]. Therefore, CCIC could be implicated in the dissemination of CMV, and their appearance in peripheral blood could be a marker of organ damage.

The purpose of our study was to examine the presence and quantity of CCIC in marrow transplant patients with asymptomatic CMV infection and CMV pneumonia.

Patients and Methods

Patients with CMV pneumonia or asymptomatic CMV infection defined as CMV antigenemia after marrow transplantation between May 1993 and June 1996 were eligible for the study. From the in-dwelling Hickman catheter, 20 mL of heparinized blood was drawn within 72 h of diagnosis of pneumonia or asymptomatic
infection. The mononuclear cell fraction was isolated by density gradient centrifugation using Histopaque (Histopaque 1077; Sigma, St. Louis).

Granulocytes were isolated by resuspension of the remaining pellet and subsequent dextran sedimentation. For the testing of antigenemia, cytospin smears of the mononuclear and granulocyte fractions were prepared from a final solution of 100 μL containing 1.5 × 10^6 cells/mL. Slides were fixed in 5% formaldehyde/NP-40 [12], air-dried, and stored at −20°C. For all patients, 10 slides each were screened for the presence of CCIC by staining with monoclonal antibodies (MAbs) against CMV protein pp65 (C10/11, provided by Biotest Diagnostic, Denville, NJ) and immunoperoxidase detection. We chose pp65-specific MAbs because of the higher sensitivity for screening compared with MAbs specific to the immediate-early antigen [13]. Briefly, slides were thawed, washed in PBS, incubated with MAb (1:10 in PBS), washed twice in PBS, incubated with peroxidase-labeled anti-mouse antibodies (Dako P161; Dako, Carpinteria, CA), washed twice, and incubated with acetate-buffered 3-amino-9-ethyl-carbazole (Sigma) [12]. Counterstaining was performed with Mayer’s hematoxylin (Accustain; Sigma). CCIC were identified by their microscopic appearance of ~35–45 μm, a kidney-shaped nucleus, and positive nuclear and cytoplasmic staining with MAb against pp65. In addition, slides of patients with CMV-positive cells were stained with PAL-E MAB (Biodesign International, Kennebunk, ME) diluted 1:100 in PBS and detected by immunoperoxidase to confirm their origin as endothelial cells.

Negative (peripheral blood of CMV-seronegative persons) and positive controls (granulocytes from patients with known antigenemia) and heart sections for endothelial cells were prepared and stained in parallel.

Staining results were classified as negative if no CCIC were present and as positive if at least one of these cells was present in 10 slides. Results were quantified by counting the number of positive CCIC per 10 slides (~1,000,000 cells, assuming a cell loss of 35% in preparation).

PCR of CMV DNA was performed on 1.5 × 10^5 peripheral blood leukocytes using base extraction–amplifying DNA from the fourth exon of the IE-1 gene, sensitive enough to detect >10 copies of CMV genome target [14]. Antigenemia testing was done using formaldehyde/NP-40 fixation and immunofluorescence staining [1].

The diagnosis of CMV pneumonia was based on the presence of pulmonary infiltrates and isolation of CMV by BAL or lung biopsy. Copathogens were defined as pathogens isolated by BAL or lung biopsy, including invasive fungal infections (Aspergillus, Mucor), respiratory viruses (respiratory syncytial virus [RSV], influenza or parainfluenza virus), and bacterial pulmonary pathogens. Antiviral treatment with ganciclovir was initiated either at time of first antigenemia (at ≥2 positive cells/slide), at time of positive blood cultures, or at documentation of CMV disease, whichever occurred first [1]. In 2 patients, prophylactic ganciclovir treatment was initiated with engraftment [1].

The associations between detection of endothelial cells, antigenemia (as a continuous variable), and CMV infection or disease (categorized into 3 groups: antigenemia only, CMV pneumonia, and CMV pneumonia with copathogen) were evaluated in a log linear regression model with SPSS 6.1.1 (SPSS, Chicago). Variables were introduced in a stepwise fashion on the basis of likelihood ratios; entry and removal levels of significance were .05 and .1, respectively.

Results

Thirty-six patients were included in this study (23 male, 13 female; median age, 32 years [range, 4–62]). Twelve patients had acute leukemia (lymphocytic, 5; nonlymphocytic, 7), 13 had chronic myelogenic leukemia, 6 had myelodysplastic syndrome, 3 had non-Hodgkin’s lymphoma, multiple myeloma, or Hodgkin’s disease, and 2 had nonmalignant diseases. Ten patients had a matched related donor, 10 had a related mismatched donor, 14 had an unrelated donor, and 2 had received an autologous transplant.

Of 36 patients, 16 had CMV pneumonia (6 without copathogens, 10 with copathogens) and 20 had asymptomatic antigenemia. Pulmonary copathogens included Aspergillus species (4 patients) and Mucor species, herpes simplex virus, RSV, parainfluenza virus, multiple bacterial pathogens, and Epstein-Barr virus–lymphoproliferative disease (1 each).

CCIC could be detected in 22 (61%) of 36 patients. In most of the slides, cells had intensive nuclear and cytoplasmic staining with pp65 MAB. Few cells had only nuclear staining, but these cells were identified only in patients who also had cells with the typical staining pattern. In addition, many CCIC were surrounded by leukocytes in contact with the cell membrane (data not shown). In contrast to previous reports [6], CCIC did not stain with factor VIII MAB. However, all slides stained positive with PAL-E MAB.

CCIC were detected in 5 of 6 patients with CMV pneumonia without copathogen and in 7 of 10 patients with CMV pneumonia and copathogens. Of patients with asymptomatic CMV infection, 10 (50%) of 20 patients with antigenemia at different

<table>
<thead>
<tr>
<th>Table 1. Incidence and frequency of CMV-infected endothelial cells in patients with CMV pneumonia and asymptomatic infection.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigenemia*</td>
</tr>
<tr>
<td>------------------------------------</td>
</tr>
<tr>
<td>Asymptomatic antigenemia</td>
</tr>
<tr>
<td>Incidence 1</td>
</tr>
<tr>
<td>Median (range)</td>
</tr>
<tr>
<td>CMV pneumonia with copathogen</td>
</tr>
<tr>
<td>Incidence 1</td>
</tr>
<tr>
<td>Median (range)</td>
</tr>
<tr>
<td>CMV pneumonia with copathogen</td>
</tr>
<tr>
<td>Incidence 1</td>
</tr>
<tr>
<td>Median (range)</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Median (range)</td>
</tr>
</tbody>
</table>

* No. of positive granulocytes/slide.

1 No. of CCIC/10 slides, each prepared using 1.5 × 10^6 mononuclear cells.

1 No. of patients (%) with any positive CCIC/group.
Table 2. Patients with different presentations of CMV disease and antigenemia.

<table>
<thead>
<tr>
<th>Day relative to transplant</th>
<th>CMV isolated</th>
<th>CCIC</th>
<th>Antigenemia</th>
<th>PCR</th>
<th>Therapy/outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1: pneumonia with high antigenemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−1, 13</td>
<td>Urine</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>18</td>
<td>—</td>
<td>ND</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>26</td>
<td>Blood, throat</td>
<td>5</td>
<td>35</td>
<td>+</td>
<td>Ganciclovir</td>
</tr>
<tr>
<td>32</td>
<td>BAL</td>
<td>7</td>
<td>834</td>
<td>+</td>
<td>Ganciclovir + IVIG</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>704</td>
<td>+</td>
<td>Ganciclovir</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Patient 2: pneumonia with low antigenemia level</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>—</td>
<td>ND</td>
<td>0.5</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>Blood</td>
<td>ND</td>
<td>28</td>
<td>+</td>
<td>Ganciclovir</td>
</tr>
<tr>
<td>35</td>
<td>—</td>
<td>ND</td>
<td>5</td>
<td>+</td>
<td>Ganciclovir</td>
</tr>
<tr>
<td>43</td>
<td>BAL (and PIV III)</td>
<td>0</td>
<td>2</td>
<td>+</td>
<td>Ganciclovir + IVIG</td>
</tr>
<tr>
<td>60</td>
<td>—*</td>
<td>ND</td>
<td>2</td>
<td>+</td>
<td>Died</td>
</tr>
<tr>
<td>Patient 3: high antigenemia, no pneumonia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>—</td>
<td>ND</td>
<td>1.0</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>32</td>
<td>—</td>
<td>ND</td>
<td>0.5</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>40</td>
<td>Blood</td>
<td>0</td>
<td>38</td>
<td>+</td>
<td>Ganciclovir</td>
</tr>
<tr>
<td>53</td>
<td>Blood</td>
<td>4</td>
<td>414</td>
<td>+</td>
<td>Ganciclovir</td>
</tr>
<tr>
<td>67</td>
<td>Blood</td>
<td>1</td>
<td>106</td>
<td>+</td>
<td>Ganciclovir</td>
</tr>
<tr>
<td>81</td>
<td>—</td>
<td>ND</td>
<td>0.5</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>139</td>
<td>Blood</td>
<td>ND</td>
<td>54</td>
<td>+</td>
<td>Developed CMV pneumonia, resolved</td>
</tr>
</tbody>
</table>

NOTE. PCR, polymerase chain reaction; ND, not determined; IVIG, intravenous immune globulin; BAL, bronchoalveolar lavage; PIV, parainfluenza virus.
* Autopsy performed, culture and histology negative for CMV.

levels had CCIC in the peripheral blood (table 1). The proportions of positive results were not significantly different between these groups ($P > .4$), nor were the numbers of endothelial cells different (Kruskal-Wallis, $P = .2$). Detection of CCIC was slightly more frequent in patients with high antigenemia, but this was not statistically significant ($P > .15$) (table 1), nor was the number correlated to antigenemia.

CMV pneumonia occurred in several patients with low antigenemia and without detection of endothelial cells (e.g., table 2, patient 2). CMV DNA PCR was done only qualitatively, so virus load could not be measured by this method.

Of 20 patients with antigenemia and no disease at time of study, 4 later had CMV pneumonia (80–180 days after the study, after discontinuation of ganciclovir treatment). Three of these 4 patients had CCIC detectable at the time of the study.

Serial observations were obtained for 2 patients (table 2, patients 1 and 3). In these patients, the occurrence of endothelial cells was related to the level of antigenemia and its time pattern. No obvious difference in outcome of CMV pneumonia was seen in patients with endothelial cells compared with those without (4/12 with CCIC resolved their CMV pneumonia vs. 1/4 without).

**Discussion**

This study demonstrated that CCIC can be detected in marrow transplant recipients with CMV disease and CMV infection; however, the frequency appeared to be substantially lower than in solid organ transplant recipients and patients with AIDS. The rates of detection for CCIC were not statistically significantly different between patients with CMV pneumonia (with and without copathogens) and asymptomatic CMV infection, nor was there a strict association with the degree of virus load.

In contrast to renal transplant patients or patients with AIDS, in whom the reported numbers of CCIC were as high as 40 of 200,000 mononuclear cells [7], the numbers in our patients were considerably lower, with a maximum of 15 in 10 slides (~1,000,000 cells). The presence of CCIC in our study was less strongly associated with CMV disease than in previous studies [6, 7]. Instead, occurrence of CCIC was not infrequent in patients with asymptomatic antigenemia (10/20, 50%), and a high number of these patients did not develop overt disease (7/10 with CCIC detected) at any time during their posttransplant course.

The reason for the lower numbers of CCIC in our patients than in renal transplant or AIDS patients is not clear. However, there are several possible explanations. First, it is possible that representative patients with particularly high numbers of CCIC were included in previous studies [6, 7] rather than consecutive patients as in our study. Second, the lower frequency of CCIC might be correlated to a lower systemic virus load that appears to be inherent in marrow transplant recipients [15]. After mar-
row transplantation, even CMV pneumonia can occur with very low systemic virus load [1, 15]. Finally, our patients differ from AIDS and solid organ transplant patients especially in regard to the earlier and more frequent preemptive use of antiviral therapy. The latter may have prevented the detection of higher numbers of CCIC.

The lack of statistical significance for the incidence and frequency of CCIC in patients with CMV disease and infection and different levels of virus load (table 1) does not rule out that differences between these groups exist. There was a trend toward a higher incidence and frequency of CCIC in patients with CMV pneumonia and those with high virus load. Indeed, if the rates observed were representative for the groups evaluated, the differences between the groups would have been statistically significant for pneumonia versus asymptomatic infection with patient numbers three times higher than evaluated here. The association of higher levels of antigenemia and detection of CCIC would have been statistically significant with twice the number of patients evaluated here (data not shown).

The fact that such a large number of patients is required to demonstrate a difference limits the practical significance of detection of CCIC as a test to differentiate between organ damage and asymptomatic infection. Instead, our results suggest that CCIC are involved in the dissemination of CMV infection rather than primarily indicating tissue damage. If only tissue damage led to the appearance of CCIC, the degree of inflammatory changes in necrotizing CMV pneumonia should induce higher numbers of CCIC cells than seen in this study. This is further supported by the similarity in outcome between patients with or without CCIC. The appearance of CCIC often in close contact with surrounding leukocytes might illustrate the bidirectional transmission between these cells and leukocytes [10] and their role in dissemination of CMV infection.

In conclusion, CCIC could be detected in patients with CMV infection regardless of end organ disease, supporting the hypothesis of their involvement in CMV dissemination. Given the high levels of CCIC in patients with asymptomatic CMV infection, the detection of CCIC in marrow transplant recipients appears to be of limited value in predicting the development of CMV disease in patients who already have antigenemia and in distinguishing between CMV as the pathogen causing pneumonia and its role as a bystander pathogen in patients with isolation of CMV and concurrent other pulmonary pathogens.

Acknowledgments

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References

Late occurrence of cytomegalovirus (CMV) disease after day 100 after bone marrow transplantation has become an increasing problem; whether a quantitative measurement of CMV DNA in plasma by polymerase chain reaction (P-PCR) could be predictive of such disease was investigated.

In a prospective study, 117 subjects undergoing allogeneic marrow transplantation were followed for 120 days with weekly CMV blood cultures, with day 35 bronchoalveolar lavage CMV cultures, with weekly CMV P-PCR, and with clinical follow-up for an additional 1–2 years. Despite preemptive ganciclovir, CMV disease occurred in 9% of subjects, with a median time of onset of 176 days. Quantitative CMV P-PCR was associated with the late development of CMV disease ($P = .01$). Of 43 subjects with positive P-PCR results, 23% developed CMV disease, but no disease occurred in the 74 subjects with negative P-PCR ($P < .001$), despite the fact that 22% had CMV isolated from lung lavage fluid and 32% had CMV isolated from blood.

Risk factors for the occurrence of human cytomegalovirus (CMV)–related morbidity after allogeneic bone marrow transplantation (BMT) can be used to guide antiviral therapy [1–5]. The most important of these factors is the occurrence of asymptomatic CMV reactivation in blood or lung [2], and infection of urine or throat has not been as predictive of later disease [1, 2]. In addition, the occurrence of graft-versus-host disease (GVHD), and other factors predisposing to this, such as age $>20$ years and HLA mismatch of donor and recipient, are important risks for CMV infection and disease [6].

With the ability to determine CMV DNA in plasma by polymerase chain reaction (PCR) [7, 8], the concentration of viral DNA can be expressed as genome copies per milliliter and can be an indication of CMV virus load. It is not clear whether the amount of measurable CMV DNA in blood after BMT is predictive of disease. In human immunodeficiency virus–infected persons, however, it has been reported that the CMV DNA concentration is significantly higher in plasma in those who eventually develop CMV retinitis [9] and in cerebrospinal fluid in those who develop CMV-related polyradiculopathy [10]. The quantity of CMV DNA in bronchoalveolar cells correlates with the presence of pneumonitis in immunsuppressed persons with CMV pulmonary infection [11]. In allogeneic BMT, the presence of CMV DNA in plasma has been associated with risk for disease [3, 4, 12] and pulmonary CMV infection [13], but the significance of the quantity of CMV DNA in plasma has not been studied. We evaluated whether quantitative plasma PCR is predictive of late CMV disease.

**Methods**

**Study population.** During 1993–1994, 117 consecutive CMV-seropositive recipients of allogeneic BMT for hematologic malignancy or severe aplastic anemia were followed for the occurrence of CMV infection and disease in a prospective study. CMV infection was determined by twice-weekly blood cultures between days 28 and 56 (day 0 = day of marrow infusion) and weekly blood cultures until day 120; by plasma PCR (P-PCR) for CMV DNA on these same blood specimens, and by bronchoalveolar lavage (BAL) on day 35 after transplant. After day 120, CMV assays were performed only on patients with clinical indications suggestive of possible CMV infection.

Ganciclovir was given preemptively, as previously described [14], to a total of 71 subjects (62%), based on CMV culture positivity in day 35 BAL fluid (collected prospectively on all subjects), on two consecutive positive blood cultures prior to day 35, or on a single positive blood culture after day 35 but before day 120. No clinical decision was made on the basis of CMV P-PCR result. Thirty-six patients (31%) were treated because BAL fluid was positive for CMV; among the 81 BAL fluid CMV-negative subjects, an additional 35 were treated with ganciclovir for positive CMV blood cultures. The 117 subjects analyzed had a median number of plasma PCR tests/subject of 9, with a range of 2 to 37 samples/subject, and all subjects had at least 1 assay at or near the onset of disease (1 patient had 2 tests, 1 had 3 tests, and all the others had $\geq4$ tests). CMV disease was diagnosed at City of Hope as defined by the presence of CMV in patients showing evidence of pneumonitis or gastroenteritis [14].

**Blood and BAL fluid culture.** Blood leukocytes and BAL fluid were cultured as previously described on MRC-5 shell vials [1,
Results

**Occurrence of CMV disease.** CMV disease occurred in 10 (23%) of 43 PCR-positive marrow transplant recipients and none of 74 PCR-negative subjects ($P < .001$ by Fisher’s exact test). There were 3 cases of CMV disease in patients positive for CMV in BAL fluids and 7 in patients with negative BAL fluid results (table 1). There were 8 cases of CMV pneumonitis, 1 on day 56 after transplant in a person not qualified to receive preemptive ganciclovir and 7 cases occurring after day 150 (days 156, 171, 181, 182, 345, 408, and 623). Of these late cases, all patients had received preemptive ganciclovir, 3 on the basis of positive result in BAL fluids and 4 on the basis of CMV-positive blood culture. In addition, there were 2 cases of CMV gastroenteritis, both occurring in persons not qualified for early ganciclovir, and the onsets of these diseases were days 30 and 79. The median time to CMV disease was day 176 after transplant. In summary, most CMV pneumonitis occurred late after marrow transplant in persons who had already been treated preemptively with ganciclovir. Some early disease occurred due to failure of culture methods to detect infection, as has been previously described [1, 2].

**CMV load and clinical infection.** As shown in figure 1A, the CMV P-PCR measurement correlated with the occurrence of disease. When the highest level of CMV DNA was compared for the group with and without CMV disease, there was a significant difference between the median values ($6.0 \times 10^4$ vs. $0.3 \times 10^4$ genome copies/mL of plasma; $P = .01$ by Wilcoxon rank sum test). Table 1 shows the relationships between CMV P-PCR results and asymptomatic CMV infection in lung, CMV-positive blood culture, and CMV disease. The median time to first positive PCR result (34 vs. 41 days) was significantly shorter in the group with lower levels of CMV plasma DNA ($<10^4$ vs. $\geq 10^6$ genome copies/mL of plasma; $P = .04$ by Wilcoxon rank sum test). The rate of occurrence of CMV-positive result in BAL fluid was significantly higher in those with positive than in those with negative P-PCR results (47% vs. 22%), which confirms a previous observation [13]. There was no disease in the 74 subjects negative by CMV P-PCR, despite the fact that 22% had detectable CMV in BAL fluid on day 35 after transplant and 32% had positive blood cultures. Since it was the trigger for early ganciclovir therapy, progressive CMV infection may have been aborted before it could be detected in the plasma. The group with positive PCR results

### Table 1. Relationship of CMV genome copies in plasma to CMV infection and disease after marrow transplantation.

<table>
<thead>
<tr>
<th>PCR result</th>
<th>Negative $(n = 74)$</th>
<th>$&lt;10^4$ copies/mL $(n = 27)$</th>
<th>$\geq 10^6$ copies/mL $(n = 16)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to positive PCR, days</td>
<td>—</td>
<td>$34^*$ (21–83)</td>
<td>$41^*$ (21–144)</td>
</tr>
<tr>
<td>CMV-positive in BAL fluid</td>
<td>16 (22%)</td>
<td>16 (59%)</td>
<td>4 (25%)</td>
</tr>
<tr>
<td>CMV-positive blood culture</td>
<td>7</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>CMV disease</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>CMV-negative in BAL fluid</td>
<td>58 (78%)</td>
<td>11 (41%)</td>
<td>12 (75%)</td>
</tr>
<tr>
<td>CMV-positive blood culture</td>
<td>17</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>CMV disease</td>
<td>0</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Total CMV disease</td>
<td>0</td>
<td>3 (11%)$^*$</td>
<td>7 (44%)$^*$</td>
</tr>
</tbody>
</table>

**NOTE.** PCR, polymerase chain reaction. BAL, bronchoalveolar lavage. Data are no. of subjects (%).

* Significantly different by Wilcoxon rank sum test: $P = .04$.

$^*$ Significantly different by Fisher’s exact test: $P = .02$.

14]. The shell vials were incubated overnight and then stained for CMV major immediate-early antigen by use of Bartels (Baxter Diagnostics, McGaw Park, IL) or LIGHT Diagnostic (Chemicon, Temecula, CA) antibody reagents.

**CMV plasma PCR.** Plasma was collected from heparinized blood, filtered, and stored at $-20^\circ$C until processed, and then the DNA was isolated and amplified by methods previously described [13, 15]. PCR detection and quantitation was done by use of an ELISA-based plate assay as described [15].

**Statistical analysis.** Proportions were compared by Fisher’s exact test or the Mantel-Haenszel $\chi^2$ test when adjusting for pre-emptive ganciclovir treatment. For subjects having a positive P-PCR result, Wilcoxon rank sum tests were used to test for a difference in median number of genome copies per milliliter of plasma between those with and without CMV disease. The highest number of genome copies (occurring prior to CMV disease, if applicable) was used for these analyses and to test for a difference in time to first positive PCR result between subjects with $<10^4$ genome copies and those with $\geq 10^4$ genome copies/mL of plasma. Univariate and stepwise logistic regression analyses were used to determine the most significant predictors of CMV disease. Candidate variables included in the logistic model were the number of genome copies per milliliter of plasma, BAL fluid results, blood culture results, the occurrence of acute or chronic GVHD, and the total amount of ganciclovir received by the subject. Thirteen subjects (11%) died before day 120 (7 were PCR-negative and 6 were PCR-positive). To take into account time to disease and to adjust for the early deaths, this analysis was repeated by using Cox regression. All tests were carried out at a two-sided significance level of .05.
and <10^4 genome copies/mL of plasma had a significantly reduced rate of disease compared with that of subjects with ≥10^4 genome copies of CMV DNA/mL of plasma (11% vs. 44%; \( P = .02 \) by Fisher’s exact test). This result remained the same after adjusting for preemptive ganciclovir treatment by use of the Mantel-Haenszel \( \chi^2 \) test. Interestingly, 21 subjects had a maximum number of genome copies per milliliter before the start of ganciclovir treatment and 19 during treatment. In the 7 subjects with ≥10^4 genome copies/mL who eventually became ill, all had CMV pneumonitis or gastroenteritis despite preemptive ganciclovir, at a median time of 181.5 days.

When the results were summarized in a contingency table, with the presence of CMV disease used as the reference standard, the sensitivity of any positive CMV P-PCR result was 100%, with specificity of 69%, positive predictive value of 23%, and negative predictive value of 100%. With a cutoff for CMV P-PCR positivity of ≥10^4 genome copies/mL, the sensitivity of the CMV P-PCR assay was 70%, the specificity 73%, the positive predictive value 44%, and the negative predictive value 89%. As noted above, the occurrence of CMV DNA in plasma was significantly associated with eventual CMV disease. However, the rate of positive culture in BAL fluid or in blood was not significantly associated with CMV disease (\( P > .20 \) for both by Fisher’s exact test). The diminished value of these tests for prediction of disease was presumably due to the fact that both tests were used to trigger the start of antiviral therapy, which would decrease disease occurrence.

By use of either a univariate or stepwise logistic regression analysis, the only variable that significantly predicted the probability of developing CMV disease in this group was the number of CMV DNA genome copies per milliliter (\( P = .02 \), by use of a log transformation of the highest number of genome copies) (figure 1B). On the other hand, BAL positivity, blood culture positivity, total amount of preemptive ganciclovir treatment, and occurrence of acute or chronic GVHD were not significantly associated with late CMV disease. The logistic curve does not ascertain the risk of any single subject developing CMV disease when the CMV load is known, but it does illustrate the contribution of increasing CMV burden to the risk of CMV disease occurrence.

**Discussion**

The ability to measure virus load promises to change the way antiviral drug therapy is used. With regard to CMV infection after BMT, in which therapy is already based on risk assignment, there is the potential for virus load determination to further refine this treatment strategy. It has been demonstrated that increased CMV DNA in the plasma of persons with AIDS is associated with a risk for CMV disease of the central nervous system [9, 10]. Studies are in progress to determine whether early treatment based on risk might be a more precise method for prevention of CMV retinitis. In allogeneic BMT, although qualitative PCR assays have been used for patient
management [3, 4], there has been no such evidence that quantitative plasma CMV DNA measurements can be used to guide antiviral prophylaxis. More importantly, there is no available risk factor for late-onset CMV disease in this population.

In the group studied here, early ganciclovir use based on blood or pulmonary CMV culture results was associated with disease in 9% of subjects, most of whom became sick late after discontinuation of ganciclovir. Disease occurred significantly more frequently in those with the highest CMV DNA plasma levels, providing further evidence that the ability of the virus to replicate in the host determines pathogenesis of disease. This is the first demonstration that the absolute value of plasma CMV DNA actually correlates with disease in this patient population. Presumably, then, the level of plasma CMV DNA is a surrogate marker for host resistance to infection. Of note, the highest levels of CMV DNA occurred at a median time of 41 days after marrow transplantation, despite the fact that the median time to all CMV disease was day 176. This discrepancy in time between peak CMV DNA in plasma and onset of disease was most likely due to the intervening use of ganciclovir, which cleared detectible infection, and to the recurrence of infection after cessation of therapy. Thus, the plasma CMV DNA level defines patients who not only have an early inability to limit CMV infection but also remain at continued high risk for disease despite ganciclovir therapy, presumably because of inability to develop CMV-specific immunity during the interval of treatment.

A mathematical model can be made that indicates the association of this increased CMV burden with the risk of eventual CMV disease. Of interest, in the era of early ganciclovir use, neither CMV blood cultures during days 28–120 after BMT, prospective day 35 BAL fluid CMV culture, nor preemptive ganciclovir treatment itself were predictors for late CMV disease. CMV positivity was detected in blood or BAL fluid in 22%–32% of P-PCR–negative subjects. We attribute these results to the fact that only a positive culture of BAL fluid or blood triggered ganciclovir therapy, reducing the risk of detecting CMV in plasma if the infection was at low levels. More than 40% (see table 1) of the blood culture–positive subjects never had a positive P-PCR test, suggesting that P-PCR might not be sufficiently sensitive for monitoring patients early after transplant. Prospective studies are needed to determine whether monitoring CMV DNA load in plasma could be used for risk assignments to begin or extend ganciclovir or other anti-CMV therapy in hopes of preventing late CMV disease after BMT. As these tests become more readily available, it is possible that guidance of treatment with CMV DNA plasma assays will lead to a more rational use of antiviral prophylaxis in this population.

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Evolution of Mutations Conferring Multidrug Resistance during Prophylaxis and Therapy for Cytomegalovirus Disease

Sunwen Chou, Gail Marousek, Susan Guentzel,
Stephen E. Follansbee, Margaret E. Poscher,
Jacob P. Lalezari, Richard C. Miner,
and W. Lawrence Drew

In a human immunodeficiency virus–infected subject, cytomegalovirus (CMV) isolated 9 months after the patient began oral ganciclovir prophylaxis was resistant to ganciclovir and cidofovir and contained mutations in both UL97 and Pol coding regions. At 1 year, retinitis developed, which progressed despite intravenous ganciclovir followed by foscarnet and then cidofovir. A subsequent buffy coat virus isolate was resistant to all three drugs and contained new mutations in UL97 and Pol. By individually transferring the observed mutations to laboratory strain AD169, it was shown that a mutation at codon 603 of UL97 conferred resistance to ganciclovir, a mutation at codon 412 of Pol conferred resistance to both ganciclovir and cidofovir, and a mutation at codon 802 of Pol conferred resistance to ganciclovir and foscarnet. This case illustrates the development of multidrug resistance during prolonged exposure to antiviral therapy for CMV and cross-resistance arising from point mutations in the CMV Pol gene.

Materials and Methods

Case report. A 42-year-old human immunodeficiency virus (HIV)–infected subject, with a CD4 cell count of 41/µL but no history of AIDS-defining opportunistic infections, was enrolled in a study of oral ganciclovir (1 g three times daily) for prevention of CMV disease. Initially there was no overt CMV disease, and an ophthalmologic examination was negative for retinitis. Study drug was twice interrupted because of neutropenia (months 2 and 3). Urine cultures were positive for CMV at baseline and intermittently thereafter (figure 1). His CD4 cell count fell to 3/µL at month 12. Thirteen months after the patient started oral ganciclovir, retinitis was noted in the right eye, limited to zone II. He had missed ~25% of his oral ganciclovir doses in the 2 months prior to diagnosis. Induction therapy with intravenous ganciclovir was started (10 mg/kg/day). Three weeks later, retinitis was detected in the contralateral eye, and therapy was switched to intravenous foscarnet (180 mg/kg/day). Disease progression was halted but relapsed when the patient began receiving maintenance foscarnet within 30 days of completion of the induction course. Reinduction therapy was attempted with the combination of ganciclovir (10 mg/kg/day) and foscarnet (180 mg/kg/day), followed by maintenance therapy with the combination. At week 25, no improvement was noted, and the patient was given intravenous cidofovir for ~5 weeks without complication, but by week 30 this therapy had failed. Reinduction treatment with ganciclovir (15 mg/kg/day) and foscarnet (180 mg/kg/day) was begun at week 31. His vision and overall clinical condition continued to deteriorate despite this therapy. Intravitreal foscarnet (2.4 mg) was administered to both eyes at week 41. His condition worsened, and he died at home 42 weeks after the initial diagnosis of CMV retinitis. A buffy coat CMV isolate from late in the clinical course (figure 1) was examined for drug susceptibility and genetic characteristics.

Virus isolates and susceptibility testing. Laboratory CMV strain AD169 (American Type Culture Collection, Rockville, MD) was used as a control. CMV susceptibility testing to ganciclovir, foscarnet, and cidofovir was done by plaque reduction in cell cultures [2]. The IC_{50} was determined. The IC_{50} cutoff used to define resistance was 6 µM for ganciclovir, 400 µM for foscarnet, and 2 µM for cidofovir [1, 2]. Three isolates from this subject were tested: at baseline and 9 and 19 months later (figure 1). The first 2 were urine isolates; the last was a buffy coat isolate.

Genetic analyses. The entire 2.1-kb UL97 and 3.7-kb Pol DNA sequence was determined for all 3 isolates selected for analysis. Sequencing was done from polymerase chain reaction (PCR)—
amplified templates representing overlapping regions of the respective coding regions. Templates were sequenced by use of a fluorescent dye terminator sequencing kit (AmpliTaq FS; Perkin-Elmer ABI, Foster City, CA) and the ABI 373A sequencer. Sequencing reactions used multiple sequencing primers representing both DNA strands to be sequenced and internal to the primers used to prepare templates [3, 4]. Sequence data were aligned with existing UL97 and Pol sequences, and all apparent deviations from reference sequences were confirmed by repeated sequencing reactions covering the same codons. Mutations in UL97 and Pol were defined as sequence changes from reference strains AD169 and Towne (sequences available in Genbank) or other known drug-susceptible clinical isolates in the existing database [1, 3–6].

Marker transfer experiments. The UL97 and Pol mutations identified in the multiresistant late clinical isolate (figure 1) were individually transferred by homologous recombination to laboratory strain AD169. The Cys-to-Trp mutation at codon 603 of UL97 (W603) and the Leu-to-Met mutation at codon 802 of Pol (M802) were transferred by use of minor modifications of a method previously published [3]. The mutant DNA fragment was 1.0 kb in length and was prepared by PCR amplification from the clinical isolate with primer pairs CPT1088 (5'-ACGGTGCTCACAGTCGTCGATGAT-3') and CPT2096 (5'-CGTCAAGGTCCTCTTGCCA-3') for W603 and Pol2566 (5'-CTATGGTTCCAGATGTGGGC-3') and Pol3550 (5'-CGTTCCTTCAGAGACCATCATCATCATCAT-3') for M802. Supernatant virus from the initial transfection was plaqued once under drug (20 μM ganciclovir for W603 and 250 μM foscarnet for M802). Plaques were picked 1–2 weeks later and screened for the presence of the desired mutant sequence, and plaques testing positive were picked for two further rounds of plaque purification without drug.

The Phe-to-Cys mutation at codon 412 of Pol (C412) was transferred by an infection-transfection procedure. Infectious AD169 extracellular virus (7 × 10^4 pfu) was ultracentrifuged, and the pelleted virus was mixed with 12 μL of the transfection reagent (Lipofectamine; Life Technologies GIBCO-BRL, Gaithersburg, MD) and the DNA fragment containing the mutation to be transferred (2 μg in 50 μL of serum-free medium). The 1.1-kb DNA fragment was PCR-amplified from the clinical isolate with the primer pair Pol1214 (5'-CGCCGTGTCTGTCACGCCG-3') and Pol2316 (5'-TGGTGTTGAACAGATCGCGCA-3') Genetic characterization of the recombinants consisted of sequencing of the entire UL97 and Pol coding regions, and each recombinant was tested for susceptibility to ganciclovir, foscarnet, and cidofovir in parallel with the parent AD169 strain.

**Results**

**Drug susceptibility of serial CMV isolates.** Viruria was intermittently detected while the patient was receiving oral
ganciclovir prophylaxis (figure 1). The baseline CMV isolate was found to be susceptible to all three drugs. After 9 months of oral ganciclovir, and with no exposure to cidofovir, an isolate was obtained that was resistant to both ganciclovir and cidofovir. The cidofovir IC_{50} of 2.8 μM was increased 7-fold over baseline but not much above the level defined for resistance (2.0 μM). However, the ganciclovir IC_{50} of 36 was high compared with those of most other resistant clinical isolates [6]. A later buffy coat isolate obtained after prolonged drug treatment was highly resistant to ganciclovir and cidofovir and resistant to foscarnet as well (figure 1).

**Sequence analysis of clinical isolates.** The complete UL97 coding sequence of the baseline susceptible clinical isolate showed amino acid change from strain AD169 at only 3 codons (68, 131, and 244). The isolate at 9 months was identical in UL97 amino acid sequence to the baseline isolate except for the presence of a mutation at codon 592 (TGC-to-GGC or Cys-to-Gly, G592) previously reported in another ganciclovir-resistant clinical isolate [6]. The third isolate showed amino acid changes at codons 125 (Leu-to-Gln) and 469 (His-to-Tyr), both previously encountered by us in susceptible clinical isolates, and at codon 603 (TGC-to-TGG or Cys-to-Trp, W603), not previously noted in susceptible clinical isolates.

The complete Pol coding sequence of the baseline susceptible clinical isolated showed amino acid differences from strain AD169 only at codons 628 (Pro-to-Leu), 655 (Ser-to-Leu), 685 (Asn-to-Ser), 884 (Ser inserted), and 885 ( Ala-to-Thr, T885). The Pol sequence of the isolate at 9 months was identical to the baseline isolate except for a mutation at codon 412 (TTT-to-TGT or Phe-to-Cys, C412). The last isolate had a Pol sequence identical to that of the 9-month isolate, except that it also contained a second mutation at codon 802 (CTG-to-ATG or Leu-to-Met, M802).

Comparative analysis of sequences from the 3 clinical isolates suggested that the G592 and W603 mutations from strain AD169 each conferred ganciclovir resistance, the C412 mutation in Pol conferred at least cidofovir resistance, and the M802 mutation in Pol conferred at least foscarnet resistance. Because of a number of possible interpretations of the cross-resistance properties of the Pol mutations, each mutation observed in the last multiresistant isolate was separately transferred to laboratory strain AD169 to test its functional effect in isolation.

**Analysis of recombinant viruses.** Three recombinant viruses were isolated and analyzed after three rounds of plaque purification. They had the mutations and susceptibility data shown in table 1. All recombinant viruses appeared to grow and passage normally in cell culture. The recombinant virus T348-11-4-11 had a UL97 coding sequence identical to that of strain AD169 and a Pol coding sequence that contained the C412 mutation but was otherwise identical to that of strain AD169. The recombinant virus T516-4-10-1 had a UL97 sequence identical to that of strain AD169 and a Pol coding sequence that contained the M802 mutation. This recombinant also contained some other amino acid differences from strain AD169 that were present in the baseline drug-susceptible clinical isolate from this subject. These were the insertion of a Ser codon at 884 and an Ala-to-Thr change at codon 885 (T885). The recombinant virus T490-5-2-1 had the UL97 amino acid sequence of strain AD169 except for the W603 mutation. The Pol sequence was identical to that of strain AD169.

**Discussion**

The evolution of this case was remarkable for several features. An initially susceptible virus became resistant after 9 months of oral ganciclovir prophylaxis for CMV in an AIDS patient, with the virus developing cross-resistance to cidofovir. This was followed by overt retinitis, which responded suboptimally to intravenous ganciclovir, foscarnet, and cidofovir. A later virus isolate had become resistant to all three antiviral drugs.

Mutations in the CMV UL97 phosphotransferase gene, specifically at codons 460, 520, or 591–596, are observed in ~90% of ganciclovir-resistant clinical CMV isolates, with mutations at codons 460, 594, and 595 being the most common [1, 3–11]. In this case, we have shown experimentally that the W603 mutation also confers ganciclovir resistance. Some other mutations in the codon 597–607 range may have a similar effect [1, 7]. Resistance to foscarnet or cidofovir is not expected to result from mutations in UL97, as illustrated by the W603 recombinant virus (table 1).

Information on the character and frequency of Pol resistance mutations in clinical CMV isolates is still limited [1, 4, 5, 7]. Because of complexity introduced by interstrain variation and possible cross-resistance, marker transfer experiments, in which putative resistance mutations are individually transferred to well-defined laboratory strains of CMV by homologous recombination, are important in determining the phenotypic correlates of specific Pol mutations. Earlier reports involving laboratory-derived CMV mutants indicated that dual ganciclovir and cidofovir resistance resulted from mutations at codons 987 [12], 501, and probably 412 [13].

In clinical isolates, Pol mutations contribute to and occasionally may be the sole basis for ganciclovir resistance [4]. Some mutations result in cross-resistance. For example, the C412 mutation conferred resistance to both ganciclovir and cidofovir (table 1) in a subject who had not received cidofovir. Mutations

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**Table 1.** Drug susceptibilities of AD169 recombinant viruses.

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Mutation</th>
<th>Ganciclovir</th>
<th>Foscarnet</th>
<th>Cidofovir</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD169</td>
<td>None</td>
<td>3.2</td>
<td>60</td>
<td>0.27</td>
</tr>
<tr>
<td>T348-11-4-11</td>
<td>Pol C412</td>
<td>11.6</td>
<td>150</td>
<td>2.7</td>
</tr>
<tr>
<td>T516-4-10-1</td>
<td>Pol M802</td>
<td>11.3</td>
<td>650</td>
<td>0.49</td>
</tr>
<tr>
<td>T490-5-2-1</td>
<td>UL97 W603</td>
<td>25.5</td>
<td>265</td>
<td>0.56</td>
</tr>
</tbody>
</table>
at Pol codons 700 and 715 resulted in decreased susceptibility to foscarnet when transferred to strain AD169 [5]. Here, the M802 recombinant was found to be resistant to both ganciclovir and foscarnet, representing the first Pol mutation shown to confer ganciclovir-foscarnet cross-resistance, a clinically significant feature.

The present findings, and other preliminary data [1, 4], suggest that Pol resistance mutations in clinical isolates tend to occur at codons 380–540 (regions IV to A), 696–845 (regions II to III), and 987 (region V). Several mutations in the codon range 380–540 are associated with ganciclovir-cidofovir cross-resistance, and mutations at or near codon M802 may indicate ganciclovir-foscarnet cross-resistance. Thus, when choosing alternative drugs for those who have received prolonged anti-CMV therapy, susceptibility testing or genetic analysis of Pol may be needed to guide the selection.

References

T Cell Subsets and Cytomegalovirus Retinitis in Human Immunodeficiency Virus–Infected Patients

Mei-Ling Tay-Kearney,* Cheryl Enger, Richard D. Semba, Walter Royal III, James P. Dunn, and Douglas A. Jabs

A case-control study was done to investigate the relationship between T cell subsets and cytomegalovirus (CMV) retinitis in human immunodeficiency virus (HIV)–infected subjects with or without CMV retinitis and CD4+ cell counts of <0.050 × 10^9/L. Cell surface markers on peripheral blood lymphocytes were evaluated using flow cytometry. Patients with CMV retinitis had significantly lower levels of CD8+ cells (median: 0.152 × 10^9/L) compared with levels for controls (median: 0.296 × 10^9/L, P < .001). Significant down-regulation of costimulatory molecule CD28+ and lymphocyte function–associated antigen-1 (LFA-1) expression was observed in patients versus controls (CD28+: 0.048 × 10^9/L vs. 0.143 × 10^9/L, P < .001; LFA-1: 0.238 × 10^9/L vs. 0.400 × 10^9/L, P < .001), but no significant differences were noted for NK cells. We propose that progressive loss of the CD3+CD8+ cell subset and down-regulation of CD28 and LFA-1 accessory molecules are associated with an increased risk of CMV retinitis in HIV-infected patients.

Cytomegalovirus (CMV) disease is assuming a more significant role as people with AIDS live longer. The cumulative lifetime risk of CMV disease is 45% [1], with retinitis being its most common manifestation. CMV retinitis is the most common intraocular infection in AIDS patients and is a cause of significant morbidity. It generally occurs when CD4+ cell counts are <0.050 × 10^9/L [2].

Despite the presence of antibodies to CMV, 30%–40% of AIDS patients develop CMV disease, suggesting that cell-mediated immunity is necessary for maintaining CMV latency. This supposition is also supported in clinical and animal studies in which recovery from CMV infection is dependent on an intact cellular immunity, in particular CD8+ T lymphocytes and NK cells [3].

In addition to the progressive loss of CD4+ and CD8+ T lymphocytes in human immunodeficiency virus (HIV) infection, it was recently reported that CD28 antigen expression declines as HIV infection advances [4]. Because loss of this molecule might partly explain cytotoxic T lymphocyte anergy [5], we postulate that progressive cytotoxic/suppressor T cell (CD8 cell) loss or anergy (or both) could predispose HIV-infected persons to CMV disease. Furthermore, since it is desirable to be able to identify patients at higher risk of CMV retinitis (for primary prophylaxis), we conducted a case-control cross-sectional study on the expression of lymphocyte subset markers in peripheral blood lymphocytes from HIV-infected persons with and without CMV retinitis. We hypothesized that decreased levels of CD8+ cells, NK cells, and costimulatory molecules (CD28 and lymphocyte function–associated antigen-1 [LFA-1]) would predispose individuals with HIV to CMV retinitis.

Materials and Methods

From September 1994 through May 1995, HIV-infected patients with CMV retinitis were recruited from the AIDS Ophthalmology Clinic at Johns Hopkins Hospital. A diagnosis of CMV retinitis was made on the basis of typical ophthalmologic features. HIV-infected patients without CMV retinitis and with CD4+ cell counts <0.050 × 10^9/L were recruited as controls. Patients with visceral CMV disease were excluded from the control group.

Blood was collected for flow cytometry and a complete blood cell count. Blood for flow cytometry was transported at room temperature and prepared within 4 h of collection. Clotted specimens were discarded. Peripheral blood lymphocytes were isolated, stained, and analyzed in general compliance with Centers for Disease Control and Prevention guidelines for leukocyte immunophenotyping of HIV-infected persons [6].

Peripheral blood lymphocytes were stained with combinations of monoclonal antibodies (MAbs), fixed, and analyzed within 12 h of staining. Isotype-matched MAbs served as controls for fluorescence marker settings and identification of nonspecific staining. All MAbs were directly conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE), or to a tandem dye, phycoerythrin–cyanine 5 (5-PE-Cy5). The following MAbs were used: CD3-PE-Cy5 (Pan-T cells; Immunotech, Westbrook, ME); MsIgG1-FITC, MsIgG2a-PE, MsIgG1–Cy5 (isotype control; Immunotech);
CD45-FITC (lymphocytes, monocytes; Becton Dickinson, San Jose, CA); CD14-PE (monocytes; Becton Dickinson); CD4-FITC (helper T cell; Becton Dickinson or Olympus, Lake Success, NY); CD8-PE (cytotoxic/suppressor T cell; Becton Dickinson or Olympus); CD28-FITC, CD28-PE (costimulatory molecule; Immunotech); CD45RA-FITC (naive T cell; Becton Dickinson); CD16 CD56-PE (NK cell; Immunotech); and LFA-1–FITC (accessory, adhesion molecule; Immunotech).

Two- and three-color flow cytometry was done using Becton Dickinson FACStar Plus and FACStar Plus Research Software (LYSIS II). Flow cytometer optics were aligned and calibrated daily with Biosure chick red blood cells (Riese, San Jose, CA). For each sample, 15,000 ungated events were collected, and the lymphocyte population was selected by backgating. Quadrant analysis cursors were set from the isotype control sample so that >98% of the gated cells were double negative. These same quadrant settings were used for subsequent antibody combinations. The raw subset percentage value was corrected for nonlymphocyte events within the gate [6].

Raw data were analyzed with statistical software (SAS Institute, Cary, NC). For many analyses, all patients with CMV retinitis were considered a single group. Categorical characteristics were compared between groups by use of Fisher’s exact test. Group means were compared using Student’s t test. Median and interquartile values were used to describe lymphocyte subset ranges, which were not normally distributed. The percent counts and absolute counts of cell surface markers were compared using the Wilcoxon rank sum test. Multivariate regression analysis was done to adjust for potentially confounding factors. Unadjusted P values are given.

**Results**

One hundred five HIV-infected persons with CD4+ cell counts <0.050 × 10^9/L were recruited prospectively, of whom 49 had CMV retinitis (patients) and 56 had HIV-infection without CMV retinitis (controls).

Demographics for the patients and controls are compared in table 1. Of the 49 patients, 22 were receiving anti-CMV treatment (ganciclovir, foscarnet, or cidofovir); 27 had newly diagnosed CMV retinitis and were not receiving treatment at the time of blood sampling. Subgroup analyses showed no significant statistical differences in demographic factors and medical histories, except for granulocyte colony-stimulating factor (G-CSF) use, for treated and untreated patients, so they were analyzed as a single group. Patients were slightly older than controls, but the 2 groups did not differ significantly in their use of acyclovir, prednisone, or cytotoxic drugs; however, fewer patients were receiving antiretroviral drugs than were controls (P = .049), and more patients than controls were treated with G-CSF (P < .001). In addition, more patients than controls had a history of Kaposi’s sarcoma (P = .019) and other opportunistic infections (i.e., Pneumocystis carinii pneumonia and cryptococcal and Mycobacterium avium-intracellulare complex infections; P = .030).

Lymphocyte subset results, comparing patients and controls, are listed in table 2. The median absolute lymphocyte count was significantly lower in the patient group than in the control group (0.517 × 10^9/L vs. 0.610 × 10^9/L, P = .018). However, both groups had a similar CD4+ cell percent (0.81 × 10^9/L vs. 1.1 × 10^9/L, P = .380) and similar median absolute CD4+ cell counts (both <0.010 × 10^9/L, P = .069).

Compared with CD8+ and CD45RA+ cell counts for controls, those for patients were lower (CD8+: P < .001; CD45RA+: P < .001). CD8+ CD45RA+ cell counts were also significantly lower in patients than controls (P < .001). Three-color analysis determined that most CD3+CD45RA+ cells (i.e., CD45RA+ cells) were also CD8+CD45RA+ cells.

CD28 antigen expression was significantly lower for CD3+ (P = <.001) and CD8+ (P = <.001) cell subsets in patients than in controls. Three-color analysis determined that most of the CD8+CD28+ cells were also CD3+CD8+CD28+ cells. T cells expressing LFA-1 were significantly lower in patients than controls (P < .001). Although the percentage of CD3–56+CD16+ cells (NK cells) was higher in patients than controls, the absolute counts were similar.

A subgroup analysis was done to determine the effect of treatment with G-CSF. The absolute number of lymphocytes

**Table 1.** Demographics and medical histories for HIV-infected subjects with (patients) or without (controls) CMV retinitis.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Patients</th>
<th>Controls</th>
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</thead>
<tbody>
<tr>
<td>No. of patients</td>
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<td>56</td>
<td></td>
</tr>
<tr>
<td>Race</td>
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<td>Black</td>
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<td>Gender</td>
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<td>MSM and IDU</td>
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<tr>
<td>Heterosexual</td>
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<td>20</td>
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<tr>
<td>Blood product use</td>
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<td>Age (years), mean ± SD</td>
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<td>Antiretroviral drug use</td>
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<td>History of other OI</td>
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<tr>
<td>No</td>
<td>29</td>
<td>50</td>
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</table>

NOTE. Data are % unless otherwise indicated. MSM = men having sex with men; IDU = injection drug use; G-CSF = granulocyte colony-stimulating factor; OI = opportunistic infections (defined as Pneumocystis carinii pneumonia and cryptococcal or Mycobacterium avium-intracellulare complex infections).

* Determined by use of Fisher’s exact test and Student’s t test.
was significantly higher in patients receiving G-CSF compared with those not receiving G-CSF (median: 0.617 × 10^6/L vs. 0.384 × 10^6/L, \( P = .023 \)). The absolute number of CD8^+ cells in patients receiving G-CSF versus those not receiving G-CSF was also significantly higher (0.308 × 10^6/L vs. 0.138 × 10^6/L, \( P = .038 \)), as was the absolute number of CD28^+ cells (0.136 × 10^6/L vs. 0.033 × 10^6/L, \( P = .010 \)) and CD8^+CD28^+ cells (0.08 × 10^6/L vs. 0.019 × 10^6/L, \( P = .027 \)). T cell counts were also significantly higher in G-CSF–treated patients. No significant group differences were found for other mononuclear subsets analyzed.

Multivivariate regression analysis showed that the differences in cell surface markers between patients and controls remained after adjusting for age, race, and antiretroviral use. The only factor that influenced the cell surface marker results was G-CSF use, which actually reduced the differences between patients and controls. Subgroup analysis also showed no significant differences in the demographics or medical histories of treated and untreated patients, except for the greater use of G-CSF in the treated group.

Cell transfer studies in CMV-infected murine models suggest that CD8^+ cytotoxic T lymphocytes are protective for CMV disease [7]. Our group of patients had significantly lower CD8^+ cell counts compared with those for the control group, and the majority of these cells were naive (CD45RA^+). Fiala et al. [8] reported that the risk for CMV disease increases when CD8^+ cell counts decline to <0.500 × 10^6/L. This finding was corroborated by Lowder et al. [9] in a retrospective study of CMV retinitis in patients with AIDS. These findings underscore the importance of CD8^+ cytotoxic T lymphocytes in the prevention of CMV retinitis.

It has been suggested that switching from CD45RA^+ (naive) to CD45RO^+ (memory) cells is an important mechanism of T cell activation in response to CMV infection. In this study, patients with CMV retinitis had lower numbers of naive cells than did the control group, suggesting that the switching of naive cells to memory cells may be inadequate to prevent CMV disease in HIV-infected patients.

CD28 antigen provides the necessary second signal for T cell activation, proliferation, and interleukin-2 production. Brinchmann et al. [5] demonstrated that in HIV-infected patients, the ability to proliferate and secrete interleukin-2 in response to T cell receptor activation was restricted to the CD8^+CD28^+ cell subset. In the current study, we found a significant decrease in T cell–expressed CD28 antigen in the HIV-infected patients with CMV retinitis, compared with HIV-

### Discussion

HIV-infected patients with CMV retinitis and controls without CMV retinitis were matched for CD4^+ cells by selecting subjects with CD4^+ cells <0.050 × 10^6/L.

Patients were more likely than controls to be white and older and to have a history of another opportunistic infection, and they were less likely to be using antiretroviral drugs and more likely to be using G-CSF. However, regression analysis showed that age, race, and antiretroviral drug use did not alter the cell surface marker results, and G-CSF use reduced the differences in some mononuclear subset measurements between patients and controls. Subgroup analysis also showed no significant difference in the demographics or medical histories of treated and untreated patients, except for the greater use of G-CSF in the treated group.

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### Table 2. Percent and absolute lymphocyte counts for HIV-infected subjects with (patients) or without (controls) CMV retinitis.

<table>
<thead>
<tr>
<th>Surface marker</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4^+ cell % (×10^6/L)</td>
<td>Absolute no. lymphocytes (×10^6/L)</td>
<td>CD4^+ cell % (×10^6/L)</td>
</tr>
<tr>
<td>CD3^+CD8^+</td>
<td>39.50</td>
<td>0.152</td>
</tr>
<tr>
<td>CD3^+CD45RA^+</td>
<td>19.43</td>
<td>0.082</td>
</tr>
<tr>
<td>CD8^+CD45RA^+</td>
<td>16.53</td>
<td>0.078</td>
</tr>
<tr>
<td>CD3^+CD28^+</td>
<td>13.38</td>
<td>0.048</td>
</tr>
<tr>
<td>CD8^+CD28^+</td>
<td>6.82</td>
<td>0.023</td>
</tr>
<tr>
<td>CD8^+CD28^+</td>
<td>25.65</td>
<td>0.120</td>
</tr>
<tr>
<td>CD3^+LFA-1^+</td>
<td>51.46</td>
<td>0.238</td>
</tr>
<tr>
<td>CD3^+CD56^+CD16^+ (NK cells)</td>
<td>12.45</td>
<td>0.063</td>
</tr>
</tbody>
</table>

**NOTE.** Data are medians; numbers in parentheses are 25th, 75th percentile values.

* Compared with controls; determined by use of Wilcoxon rank sum test.
infected controls. This loss of CD28 antigen was mainly observed in the CD8+ population of T cells, suggesting that loss of the costimulatory molecule may result in a less effective cytotoxic/suppressor response to CMV-infected cells. The greater loss of CD28 antigen expression might also explain the poorer proliferative responses of T cells isolated from HIV-infected persons with CMV retinitis compared with responses of T cells from HIV-infected persons without CMV retinitis [10]. Factors other than CD28 antigen expression may also contribute to the poorer lymphoproliferative responses observed in vitro. For example, ganciclovir, acyclovir, and zidovudine each have been reported to inhibit lymphocyte proliferation in vitro [11]. Furthermore, CMV infection itself has been reported to have immunosuppressive effects, manifested by a decreased lymphoproliferative response after mitogen stimulation and by decreased cytolysis of virus-infected cells [12]. However, lymphocytes are usually nonpermissive for CMV infection, so a direct immunosuppressive effect of the virus at the T cell level is unlikely.

Interactions between LFA-1 and intercellular adhesion molecule 1 contribute to the activation of the T lymphocyte and can augment cytolysis of virus-specific CD8+ cells [12]. In the current study, HIV-infected patients with CMV retinitis had significantly lower levels of CD3+LFA-1+ cells compared with levels for the HIV-infected controls. Loss of LFA-1 could further contribute to immunodeficiency and, thus, to an increased risk of CMV retinitis.

Animal and clinical studies have shown that NK cells play a role in CMV disease, though they generally delay but do not prevent severe disease and death [13]. In our study, there were no significant differences in the absolute numbers of NK cells in subjects with or without CMV retinitis, suggesting that the cells have a less important role in the prevention of CMV disease in HIV-infected persons with progressive immunosuppression.

A subgroup analysis of T cell subsets was done to study the effects of G-CSF in HIV-infected patients with CMV retinitis. It has been previously reported that there is a disproportionate increase in CD8+ cells during treatment with G-CSF [14]. In our study, we found significant increases in the absolute lymphocyte and CD8+ cell counts but no effect on the CD4+ cell count. The CD8+CD28− cell numbers also increased in patients treated with G-CSF. The G-CSF–initiated increase in cells bearing costimulatory molecule CD28 is consistent with the reported increase in lymphoproliferative responses to phytohemagglutinin of T cells from HIV-infected patients treated with G-CSF compared with those from untreated patients [14]. This effect of G-CSF on T cell subsets tended to minimize the differences between subjects with and without CMV retinitis.

Whether the observed changes in T cell subsets in HIV-infected patients with CMV retinitis represent a cause or effect of CMV disease cannot be determined from this study. However, in light of previously published observations and the observations from our study, one plausible hypothesis is that a loss of CD8+ cytotoxic T cells with down-regulation of CD28 and LFA-1 antigens (a change that leads to an ineffective cytotoxic response to virus-infected cells) contributes to an increased risk for CMV retinitis. Screening of HIV-infected patients with CD4+ cell counts <0.500 × 10^9/L, CD8+ cell counts <0.500 × 10^9/L, and low numbers of CD8+CD28+ and CD8+LFA-1+ cells might better identify patients at risk of CMV retinitis. Such patients may benefit from primary prophylaxis for CMV disease.

Finally, successful adoptive immuno­therapy for human viral infections requires an understanding of immune mechanisms essential for providing the host with a protective response. The observed changes in the cytotoxic cell subset profile in AIDS and CMV retinitis may also help to develop more virus-specific T cell subsets for adoptive therapy for CMV disease [15].

Acknowledgments

We thank Gordan Weigand for expert instructions given in the use and running of the flow cytometer and Diane Griffin for the use of her neurovirology laboratory.

References

Human Immunodeficiency Virus RNA Levels in US Adults: A Comparison Based upon Race and Ethnicity


Volunteers in a natural history study of human immunodeficiency virus type 1 (HIV-1) at two military medical centers were studied to determine whether plasma HIV-1 RNA levels differ among racial and ethnic groups of US adults infected with HIV-1. Cross-sectional analyses of plasma HIV-1 RNA and CD4 cell counts were done using demographic and clinical data collected during study visits. Age, gender, CD4 cell count, seroconversion status, and use of antiretroviral therapy were studied in 545 military members (46% white, 49% black, and 6% Hispanic). No association was found between HIV-1 RNA levels and race or ethnicity among infected adults for whom access to care and socioeconomic status were not confounding factors.

Minority groups within the United States are affected disproportionately by the human immunodeficiency virus type 1 (HIV-1) epidemic. In the most recent national report of leading causes of death, the ratio of age-adjusted death rates related to HIV-1 infection is 4:1 for black versus white persons [1]. Since 1991, AIDS has been the leading cause of death for black and Hispanic men and black women 25–44 years old [2]. A number of studies have found that survival time for persons with AIDS differs among racial groups [3], and a recent article suggests that CD4 cell count decline varies with race [4]. However, such comparisons are generally confounded by socioeconomic factors and unequal access to health care. Analyses of both hemophilia and homosexual cohorts have shown that the level of HIV-1 RNA circulating in the serum or plasma is a predictor of time to development of AIDS and to death [5, 6]. Whether virus load (circulating RNA level) differs among racial and ethnic groups is unknown, and virus load may affect the rate of disease progression.

The factors confounding most comparisons of racial and ethnic groups in the United States are equalized within the military system. Quality health care is accessible to all members. More specifically, HIV-1 screening and patient management are carried out according to standardized policies [7]. A long-term study of the natural history of HIV-1 infection established by the US Department of Defense (DoD) has enrolled approximately equal numbers of white and black volunteers and a smaller number of Hispanic volunteers [8]. As part of this study, HIV-1 RNA quantitation in plasma was initiated in 1995 through a single central laboratory. Herein we report on an analysis that was based on results of the initial year of testing. The analysis addresses the hypothesis that differences in HIV-1 virus load may be associated with race.

Materials and Methods

Study participants were recruited from among volunteers enrolled in a DoD protocol for investigating the natural history of HIV-1 infection in members of the US military. Inclusion criteria
were a first RNA measurement during the study period (August 1995 to August 1996), documented CD4 cell quantitation within 7 days of RNA specimen collection, and racial or ethnic grouping as non-Hispanic white, non-Hispanic black, or Hispanic.

Blood was collected during routine clinical visits to either the National Naval Medical Center or the Walter Reed Army Medical Center. Anticoagulated whole blood specimens were drawn in the morning and delivered by courier to a central laboratory (SRA Technologies, Rockville), where plasma was separated and frozen (−70°C) within 6 h of collection.

HIV-1 RNA was quantitated in plasma with a commercial reverse transcriptase–polymerase chain reaction (RT-PCR) assay which utilized internal controls of amplification efficiency (AmpliCor HIV Monitor test; Roche Molecular Systems, Branchburg, NJ). Batched specimens were thawed, and RNA was extracted from the plasma and assayed according to the manufacturer’s instructions by technicians certified in the procedure.

CD4 cell numbers were calculated from the CD3CD4 cell percent as determined by flow cytometry, and the absolute lymphocyte count was calculated from the complete blood cell count. The flow cytometry laboratories in both hospitals were active participants in the DoD flow cytometry quality assurance program [9] and also received proficiency testing from the College of American Pathologists.

Data from all patients in the natural history study were entered into a central computerized database that is secure and without patient identifiers. The subset of individuals studied here for RNA level were analyzed for age, sex, military service, use of antiretroviral drugs, and CD4 cell count (week before or 1 week after RNA measurement). Analysis of documented recent seroconversion was defined by a negative HIV-1 serology in the prior 2 years.

RNA quantitation results were log-transformed for analyses, including x² tests for comparison of categorical HIV-1 RNA or CD4 cell counts among racial groups. Nonparametric Kruskal-Wallis tests for continuous HIV-1 RNA and CD4 cell values were done, and Spearman’s correlation coefficient was used to measure the correlation between HIV-1 RNA and CD4 cell counts.

**Results**

Plasma specimens were analyzed from 545 persons during the 12-month study period (table 1). Of this group, 249 (46%) of the participants were white, 265 (49%) were black, and 31 (6%) were Hispanic. Of these people, 92% were male; 55% were <35 years of age, and 72% and 28% were seen at the naval and army medical centers, respectively. Recent seroconversion (within 2 years of negative HIV-1 serology) was documented in only 19% of those studied. Antiretroviral drug therapy had been given to 71% of the cohort. Analysis of these variables revealed no racially associated differences (table 1).

The mean ± SD HIV-1 RNA value for the 545 subjects was 4.30 ± 0.85 log RNA copies/mL. The mean RNA values for white persons, black persons, and Hispanic persons did not differ significantly (table 1; P = .83). RNA results ranged from <200 to 2,162,208 copies/mL. The distribution of results within each racial and ethnic group was similar: 7%–9% of RNA values were <1000 copies/mL, 26%–28% were 1000 to <10,000 copies/mL, 42%–45% were 10,000 to <100,000 copies/mL, and 20%–23% were ≥100,000 copies/mL (P = .97).

CD4 cell counts represented the full spectrum: 14% were <50 cells/mL, 17% were 50–199 cells/mL, 48% were 200–499 cells/mL, and 22% were ≥500 cells/mL. CD4 cell distribution was not associated with racial or ethnic classification (P = .62). RNA values and CD4 cell counts were negatively correlated for the whole cohort (r = −.54, P = .001) and for each racial group. RNA values (mean ± SD) are shown for each stratum of CD4 cell count in figure 1, and again values were found to be independent of racial or ethnic group (in each CD4 cell count stratum, P ≥ .13).

No association was found between RNA levels and age, gender, HIV-1 seroconversion status, or branch of service. Patients who had received prior antiretroviral chemotherapy tended to have higher plasma RNA levels than those previously untreated (median, 4.5 vs. 4.0 log RNA copies/mL; P < .001), consistent with the greater likelihood that patients with more advanced stages (and lower CD4 cell counts) of disease would have been treated. When RNA level was plotted against time since seroconversion (midpoint between last negative and first positive HIV-1 test) for the 110 participants with documented seroconversion (known <2-year interval from HIV-1-negative to HIV-1-positive status), no race- or ethnicity-related differences were observed.

**Discussion**

In this cross-sectional study of 545 US adults with HIV-1 infection, plasma RNA values ranged from undetectable to ≥2,000,000 copies/mL. Since the study group consisted of similar numbers of white (46%) and black (49%) subjects, HIV-1 RNA values could be compared by group with confidence. No race- or ethnicity-related difference was found in plasma RNA levels. Furthermore, although making up only 6% of the total study group, Hispanic subjects had RNA levels no different than those for members of the larger groups. The similarity in plasma RNA levels was found after controlling for CD4 cell count, use of antiviral drugs, and time since seroconversion. These findings are consistent with our previous report that HIV-1 antigenemia does not differ by racial group [10].

The RT-PCR assay used to quantitate RNA reportedly gives low values for HIV-1 subtypes A and E [11]. While infection with a non-B subtype of HIV-1 has been documented in 5% of U.S. servicemen [12], most infections appear to be acquired within the United States [13] and therefore are presumed to be subtype A. A method for determining HIV-1 serotype or subtype in newly seroconverted persons is being developed for inclusion in the DoD HIV-1 surveillance program.

The prognostic value of HIV-1 RNA levels has been shown in cohort studies with >10 years of follow-up data [5, 6], and assessment of this predictor in different racial and ethnic groups is similarly important. Comparisons among groups tend to be confounded by differences in such factors as behavioral risks,
Table 1. Characteristics of 545 subjects in the Department of Defense HIV natural history study, grouped by racial or ethnic group.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>White (n = 249)</th>
<th>Black (n = 265)</th>
<th>Hispanic (n = 31)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA (log copies/mL)</td>
<td>4.30 ± 0.85</td>
<td>4.29 ± 0.86</td>
<td>4.41 ± 0.83</td>
<td>.83</td>
</tr>
<tr>
<td>Male</td>
<td>92</td>
<td>91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;35 years old</td>
<td>53</td>
<td>57</td>
<td>45</td>
<td>.43</td>
</tr>
<tr>
<td>Medical center attended</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walter Reed</td>
<td>23</td>
<td>32</td>
<td>29</td>
<td>.08</td>
</tr>
<tr>
<td>National Naval</td>
<td>77</td>
<td>68</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>HIV status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preexisting infection</td>
<td>80</td>
<td>81</td>
<td>90</td>
<td>.40</td>
</tr>
<tr>
<td>Documented seroconversion</td>
<td>20</td>
<td>19</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Antiviral treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever received</td>
<td>73</td>
<td>69</td>
<td>71</td>
<td>.61</td>
</tr>
<tr>
<td>Never received</td>
<td>27</td>
<td>31</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>CD4 cell count (cells/mL)</td>
<td>346 ± 294</td>
<td>358 ± 260</td>
<td>325 ± 191</td>
<td>.71</td>
</tr>
</tbody>
</table>

NOTE. Data are % subjects or mean ± SD.

socioeconomic status, and access to medical care. Because of equal access to health care, similarities in economic status, and exposure to standardized education programs for behavioral modification, racial and ethnic groups within the military provide a more accurate basis for comparison.

These results have implications concerning prognosis, transmissibility, and patient therapy. To the extent that RNA levels predict clinical outcome, these data suggest that members of different racial and ethnic groups will experience disease progression at similar rates. To the extent that infectiousness is associated with levels of HIV-1 RNA in the blood [14], transmission risk per exposure should also be independent of race and ethnicity. This study provides no data on levels of viremia during acute infection; comparative infectiousness during the

Figure 1. HIV-1 RNA levels in plasma from adult subjects participating in the US military’s study of the natural history of HIV-1 infection, grouped by race or ethnicity and CD4 cell count. Data are presented as median (●), with bar spanning 10th–90th percentiles.
seroconversion period remains to be determined. Finally, to the extent that antiretroviral therapy influences the course of disease by decreasing levels of circulating HIV-1 RNA [15], clinical responses to chemotherapy may be expected to be independent of the racial or ethnic identity of a patient as well.

Acknowledgments

We thank the volunteers for their commitment to this multiyear natural history study; the nurses, clinicians, and data managers who made this study possible; and Merlin Robb for thoughtful review of the manuscript.

References

Serum Levels of Human Immunodeficiency Virus Type 1 (HIV-1) RNA after Seroconversion: A Predictor of Long-Term Mortality in HIV Infection

Kevin J. P. Craib, Steffanie A. Strathdee, Robert S. Hogg, Barbara Leung, Julio S. G. Montaner, Michael V. O'Shaughnessy, and Martin T. Schechter

A cohort of 79 homosexual men with documented dates of human immunodeficiency virus type 1 (HIV-1) seroconversion and baseline CD4 cell counts of ≥500/µL were followed for up to 11.5 years. HIV-1 RNA was measured from stored sera obtained a median of 7 months after the estimated seroconversion date. AIDS progression and mortality among the men were studied, stratified by median baseline levels of HIV-1 RNA. AIDS progression rates at 11.5 years were 69% and 34%, respectively, among those with higher versus lower than median baseline virus loads (≥3040 copies/mL; \( P = .002 \)), and mortality rates were 61% and 27%, respectively (\( P = .003 \)). Survival curves continued to diverge throughout the 11.5 years, suggesting that the future clinical course of HIV-1 infection may already be determined at the earliest phases of disease. Initiation of definitive treatment very early in HIV-1 infection may be essential.

Much evidence has been gathered recently about human immunodeficiency virus type 1 (HIV-1) RNA as a prognostic marker in people infected with HIV-1 [1–5]. These studies have demonstrated the value of early HIV-1 RNA levels as predictors of short-term clinical progression in seroconverted persons [2], of baseline HIV-1 RNA levels as predictors of longer-term clinical progression and mortality in seroprevalent persons [1], and of treatment-induced decreases in HIV-1 RNA as markers of therapeutic efficacy [5]. Some of these findings have been replicated using serum [4]. To our knowledge, no study has investigated the value of early HIV-1 RNA levels in predicting long-term progression and mortality in recently infected persons with CD4 cell levels >500/µL. We are currently investigating the relationship between early decreases in virus load and long-term mortality, seeking evidence to support very early intervention. We present preliminary findings on the relationship between baseline serum HIV-1 RNA levels and AIDS progression and mortality in 79 homosexual men with documented dates of HIV-1 seroconversion and baseline CD4 cell counts ≥500 cells/µL who were followed for up to 11.5 years.

Materials and Methods

We studied a subgroup of homosexual men in the Vancouver Lymphadenopathy-AIDS Study who were recruited between 1982 and 1984 from six general medicine practices [6]. The men were HIV-1–negative at enrollment and seroconverted between 1983 and 1991 (median follow-up: 9 years). Follow-up visits occurred biannually until 1986 and annually thereafter. At each visit, participants completed a self-administered questionnaire and a functional inquiry and underwent a physical examination. Blood specimens collected for HIV-1 antibody and laboratory testing were also cryopreserved (−20°C). A total of 79 men for whom stored sera were available from the first HIV-1 antibody–positive (baseline) visit who had baseline CD4 cell counts ≥500/µL were eligible for analysis.

HIV-1 RNA was extracted from each serum sample. Reverse transcriptase polymerase chain reaction was done using the AmpliCor HIV Monitor Kit (Roche Molecular Systems, Branchburg, NJ), which measures RNA over a range of 200 to 1 million or more copies/mL. CD4 lymphocyte counts and clinical symptoms associated with acute retroviral illness (e.g., fever, diarrhea, night sweats) were determined during the baseline visit.

Survival analysis was used to study the relationships between baseline serum RNA, time to primary AIDS diagnosis, and time to AIDS-related death. The starting point for these analyses was the estimated date of seroconversion, defined as the midpoint between the last negative and first positive antibody test. Event-free men were censored at 30 June 1996. Kaplan-Meier survival curves for progression to AIDS and death, stratified by median RNA values, were generated. Cox regression models were used to assess the independent effect of baseline RNA virus load on AIDS-related mortality, adjusting for demographic variables, subsequent antiretroviral treatment, early symptoms, and length of time since seroconversion.

Results

Most of the 79 seroconversions occurred between 1983 and 1985, prior to the availability of antiretroviral therapies. Median age at seroconversion was 32 years. Median HIV-1 RNA levels and CD4 cell counts at baseline were 3040 copies/mL (range: 0–81,600) and 769 cells/µL (range: 510–1760), respectively.
HIV-1 RNA levels were similar for men for whom the time between the estimated date of seroconversion and the date of specimen collection was greater than or less than 7 months. A total of 36 men (46%) received a primary AIDS diagnosis; 31 (39%) died during the follow-up period.

Kaplan-Meier curves for HIV-1 survival stratified by baseline RNA median values are shown in figure 1. The product limit estimate of cumulative mortality at 11.5 years was 61% among men with high baseline virus loads and 27% among those with low baseline values ($P = .003$). Very similar curves were obtained when AIDS-free survival was stratified by median RNA level (data not shown). The product limit estimate of AIDS progression was 69% at 11.5 years among men with high baseline virus loads and 34% among those with low virus loads. Men who had seroconverted with high virus loads were more than twice as likely to progress to AIDS during follow-up ($P = .002$).

The mortality risk ratio for baseline HIV-1 RNA level indicated that the risk of death was 3.2 times higher (95% confidence interval [CI]: 1.5–6.7; $P = .004$) for men with baseline RNA values above the median after adjustment for baseline CD4 cell count. Baseline CD4 cell count did not attain conventional statistical significance in this model, but men whose CD4 cell counts were below the median (769/µL) had a mortality risk ratio of 1.8 (95% CI: 0.9–3.7; $P = 0.10$).

**Discussion**

Our data confirm the results of other studies that have shown that a single determination of HIV-1 RNA in plasma [1–3, 5] or serum [4] is a strong predictor of progression to AIDS and death in HIV infection. However, while some of these studies have used overall survival as the outcome and others have used recently infected men, none to our knowledge has established the value of HIV-1 RNA levels determined soon after infection in predicting survival over an 11-year period. Mellors et al. [2] showed that plasma levels of HIV-1 RNA after seroconversion were strong predictors of CD4 cell decline and AIDS progression, but this was limited to the first 5 years following infection.

We found that in the first stages of HIV infection, as early as only 7 months (median) following seroconversion in men with CD4 cell counts $\geq 500/\mu$L, higher virus load was associated with a 3-fold increase in risk of death over the subsequent 11 years. Moreover, analysis showed that this was not a short-term effect occurring only in the period soon after CD4 cell measurement, but rather that the elevation in risk continued throughout the 11 years of follow-up. These observations led to a number of hypotheses. The first is that early virus load provides an indirect measure of early total virus burden and of the degree to which initial infection seeds the various reservoirs with HIV-1. Alternatively, differences in early virus load may reflect different host-agent interactions that will continue throughout the course of infection and which, as suggested by Ho [7], determine the equilibrium point at which the host and virus settle after the initial viremia. Baseline samples from most patients in the present study were taken several months after seroconversion and likely already reflected steady-state levels.

We used stored sera, and it is possible that some degradation of RNA occurred. Moreover, the samples were obtained from patients in very early stages of HIV-1 infection; some may even have been obtained during the acute retroviral illness. However, we did not find a difference in HIV-1 RNA levels.
among subjects for whom the interval since the estimated date of infection was above the median and those for whom the interval was below the median, and adjustment for the length of the interval did not affect the model. Nevertheless, use of sera (as opposed to plasma), possible loss of RNA, and early sampling might have caused some misclassification of virus load, which could have attenuated the estimates of risk. The elevation in mortality risk that would be observed if plasma was sampled at precisely the same time following infection would likely be higher. The fact that baseline CD4 cell counts did not attain statistical significance as independent predictors is not surprising, given that subject selection was restricted to men with CD4 cell counts ≥500/μL; thus, our ability to detect associations would be constrained.

Our data on virus load early in HIV-1 infection suggest that a patient’s future clinical course may be significantly predetermined in the initial years following infection. This supports the view that interventions at relatively advanced clinical thresholds may be too late and that it may be necessary to initiate definitive treatments far earlier [8], although no direct data confirm or refute this. However, an association between high early virus load and subsequent mortality does not necessarily mean that lowering the early virus load will lower long-term mortality. O’Brien et al. [5] have shown a beneficial effect of lowering virus load on clinical progression in more advanced HIV infection, while Kinloch-de Loës et al. [9] have demonstrated a short-term effect of zidovudine monotherapy on CD4 cell counts and progression in primary HIV infection. These data provide justification for trials of aggressive early intervention aimed at decreasing plasma virus load [10].

While only experimental evidence can be definitive about the value of very early intervention in HIV-1 infection, current small trials of aggressive early therapy either will not provide results on long-term effects or will take many years to complete if they do. In the meantime, observational data from archived cohort studies can be useful. We are currently investigating whether early decreases in virus load are associated with slower progression and longer survival over the same 11-year follow-up.

References
Dynamic Features of Human Immunodeficiency Virus Type 1 (HIV-1) Viremia: Kinetics of Cell-Free HIV-1 RNA after Therapeutic Plasma Exchange

Patrizia Bagnarelli, Marco Candela, Anna Valenza, Aldo Manzin, Laura Solforosi, Francesca Mazzola, Luca Butini, Maria Montroni, Armando Gabrielli, Pietro E. Varaldo, and Massimo Clementi

To gain insight into the variables that influence the dynamics of human immunodeficiency virus type 1 (HIV-1) viremia levels, HIV-1 RNA molecules were quantified in plasma from an infected patient undergoing therapeutic plasma exchange (TPEX). After each TPEX procedure (2000 mL of fluid exchanged per session), HIV-1 genome molecule levels dropped to 58%–63% of the basal level but rapidly reverted to pre-TPEX values (doubling time = 3.50–4.04 h). Of interest, mobilization of extravascular cell-free virions (on average, $5.15 \times 10^4$ viral genome molecules/h) had already occurred during TPEX. After three daily TPEX procedures, HIV-1 viremia rebounded to basal values, while HIV-1 proviruses and viral transcripts in peripheral blood lymphocytes constantly tested at stable levels. Overall, this study extends previous analyses of the rate of HIV-1 turnover, using an alternative approach to the use of antiretroviral drugs, and it provides, albeit indirectly, insights into the amount and dynamic features of extravascular cell-free virus.

The availability of molecular methods and strategies to assess virus load in vivo has opened new prospects for the understanding of the natural history of human immunodeficiency virus type 1 (HIV-1) infection [1, 2]. At present, these methods are of crucial help in pathogenic research and in clinical applications, including diagnosis, prognosis, and monitoring of specific antiviral therapies [3, 4]. Recent research has indicated that HIV-1 infection is a highly dynamic process [5, 6], with the turnover of infected cells and circulating virions being quite rapid during all phases of infection [7]. A steady-state level of cell-free virus in plasma is generally maintained for years, virus clearance being balanced by virus production [1]. Although this steady-state level varies from patient to patient [8], a recent study has suggested that a single measurement of virus load can predict disease progression [9]. In this context, studies aimed at improving our understanding of the variables that define or influence the dynamic features of HIV-1 viremia are of central importance for the correct evaluation of the data obtained by monitoring infected patients. For this reason, we analyzed cell-free HIV-1 RNA levels in plasma samples from an infected patient before and after removal of large amounts of virions by three consecutive daily plasmapheresis procedures (therapeutic plasma exchange [TPEX]).

Materials and Methods

The patient, a 63-year-old woman with an HIV-1-seropositive male sex partner, was known to be infected since October 1993. At the time of this study, the patient’s disease was, according to the Centers for Disease Control and Prevention classification system, class B3 [10]: she had 196 CD4$^+$ T cells/mm$^3$ of blood and had peripheral neuropathy due to a concurrent mixed cryoglobulinemia associated with hepatitis C virus infection. TPEX was done to treat neurologic complications of the disease.

The separator used in this study (Autopheresis-C Therapeutic Plasma System; Baxter, Nivelles, Belgium) was equipped with a centrifugation system that allowed cells to be separated from plasma, while the contamination of plasma by cells was prevented by filtration. The separator had a 53-mL disposable set of extracorporeal volume; fluid balance during the process was maintained by a system of three electronic scales, which allowed continuous blood-flow processing and simultaneous readjustment of plasma volume with replacement fluids. The volume of fluid exchanged in each 2-h TPEX procedure was 2000 mL, as estimated on the basis of the patient’s weight (42 kg) and hematocrit: circulating blood volume = patient’s weight in kg × 70 mL; circulating plasma volume = circulating blood volume × 1.0 – (hematocrit as decimal × 0.91) [11]. The 2000 mL of fluid that were removed in each session were replaced with 500 mL of 0.9% NaCl and 1500 mL of albumin, 5%, plus anticoagulant (sodium citrate and citric acid). In the three TPEX procedures, 59%, 55%, and 58% of plasma volume were exchanged, respectively.

Sequential plasma samples were collected before TPEX ($-22, -16, -10, -3.5, and 0$ h), at short intervals after each TPEX session (treatments were done at 0, 24, and 48 h), and once daily for 4 days after the last session. To determine the amount of cell-free virus subtracted in each session, the fluid collected with each
TPEx was measured, and aliquots were analyzed. Plasma samples were centrifuged (1300 g for 10 min) to clear platelets and cell debris; afterward, viral RNA was purified using a previously described single-step procedure [12]. Specific HIV-1 RNA copy numbers were determined by use of quantitative competitive reverse transcriptase polymerase chain reaction, following a previously described procedure [8, 12, 13].

Results

The dynamics of HIV-1 viremia from −22 h before each TPEx procedure, shortly after each TPEx procedure, and during the 4 days following cycle completion are shown in figure 1A, and the HIV-1 p24 antigen levels are shown in figure 1B. To assess the range of circadian fluctuation of plasma viremia and to prevent it from influencing the analysis of virus load shortly after treatment, the basal level was determined four times before the first TPEx session (i.e., at −22, −16, −10, and −3.5 h). The average basal level of HIV-1 RNA molecules was 5.23 ± 0.86 × 10⁵ (coefficient of variation [CV] = 16%)/mL of plasma. The quantitative data supply a kinetic picture of the cell-free virus load observed during the study (figure 1A). HIV-1 RNA copy numbers evaluated at the beginning of each TPEx procedure (0, 24, and 48 h) were 5.75, 6.23, and 5.50 × 10⁵, respectively. After an initial drop to 63%, 60%, and 58%, respectively, due to TPEx, pretreatment levels of HIV-1 RNA molecules in plasma were rapidly restored; some were even higher at some time points. Figure 2 shows the variations of HIV-1 copy numbers in plasma shortly after each TPEx procedure. Linear regression was used to obtain the best fit for four data points (i.e., variations in viremia levels observed immediately after TPEx (time 0) and 1, 2, and 3 h after treatment, with the last time point considered being that which fell within ±10% of the pretreatment level. The best function modeling the initial increase in plasma viremia was exponential (see legend to figure 2), and the slope of each curve represents the rate of exponential increase. Thus the doubling time (T2) of HIV-1 RNA copy numbers after each TPEx session was 3.50, 3.52, and 4.04 h, respectively, as determined by dividing natural logarithm, ln(2), by the slope.

After the three TPEx procedures, HIV-1 RNA copy numbers averaged 7.2 ± 2.0 × 10⁵ molecules/mL, as evaluated at 72, 96, 120, and 144 h (CV = 27.9%), and were on average 38% higher than the mean pre-TPEx level. Finally, the amount of HIV-1 RNA molecules in the 2000 mL of fluid collected during each TPEx procedure was 9.1, 4.6, and 6.0 × 10⁸ in the three daily treatments, respectively.

Discussion

Almost all untreated subjects with HIV-1 infection show detectable levels of cell-free virus in plasma. Most HIV-1 virions are produced during continuous cycles of de novo infection, replication, and cell turnover [1], and the degree of virus replication is very high in lymphoid tissues [14]. Under these conditions, the level of plasma HIV-1 viremia reflects, at any time point, the dynamics of virions replicating in circulating cells and of those diffusing from the extravascular compartment, including the contribution of virions arriving into the blood via the lymphatic drainage system. The data shown here, indicating that the extravascular compartment of cell-free virions is in balance with the plasma viremia level (and can promptly restore the pre-TPEx equilibrium), provide indirect insight about the amount of extravascular virions.
Figure 2. Variations of HIV-1 RNA molecules in plasma following therapeutic plasma exchange (TPEx). Viremia levels immediately before and immediately after each 2-h TPEx session (time = 0 and 2) are shown for first ( ), second ( ), and third ( ) treatments. Increases in viremia levels were observed shortly after each session, and viral load reverted to pre-TPEx values within 3 h. Dynamics of viremia increased after each TPEx procedure (from time 0 to 3) and were followed by exponential trend (\( \gamma = a \cdot e^{bx} \), where \( a \) is intercept on \( y \) axis at time 0, \( b \) is slope, and \( e \) is Nepero’s no.). Equations for 3 curves were as follows: first treatment, \( y = 335,150 \cdot e^{0.197x}, R^2 = .89 \); second treatment, \( y = 370,850 \cdot e^{0.197x}, R^2 = .98 \); third treatment, \( y = 325,130 \cdot e^{0.197x}, R^2 = .99 .\) Doubling times, as determined by dividing natural logarithm, \( \ln(2) \), by slope, were 3.50, 3.52, and 4.04 h after treatments 1–3, respectively.

Indeed, analysis of HIV-1 viremia after perturbation with TPEx may provide an interesting approach for studying the exchange of virions among different compartments. A recent study addressing the elimination of an artificial immune complex (IC) by plasmapheresis in rabbits highlighted the mobilization of ICs from different organs following plasma exchange, documented by increasing IC concentrations after plasmapheresis [15]. Our results suggest that a similar rapid mechanism provided for the readjustment of the viremia level after TPEx. Of note, since the amount of fluid exchanged during each TPEx procedure ranged from 55% to 59% (average, 57.3%) of plasma volume, a corresponding drop of HIV-1 viremia was expected (i.e., starting from a mean basal level of 5.83 \( \times 10^5 \) RNA copies per mL [CV = 6.28%], a level of 2.48 \( \times 10^5 \) should have been detected after TPEx). Indeed, the drop of HIV-1 RNA copy numbers ranged from 37% to 42% (average, 39.7%, corresponding to a mean value of 3.52 \( \times 10^5 \) [CV = 8.0%] RNA copies/mL; figure 2, time 0), thus suggesting that mobilization of extravascular cell-free virions was a very rapid event and had already occurred during plasmapheresis.

These data allow for the amount of HIV-1 virions arriving into the vascular compartment during the 2-h TPEx to be calculated (i.e., 1.03 \( \times 10^5 \) HIV-1 RNA molecules, the difference between the expected and real post-TPEx viremia levels, corresponding to 5.15 \( \times 10^4 \) HIV-1 RNA molecules/h). In addition, the study shows that repeated mechanical depletion of 37%–42% of cell-free HIV-1 in plasma does not significantly affect the continuous cycles of de novo infection and virus replication, since no significant modifications of HIV-1 proviral DNA and transcript levels were observed (see legend to figure 1) in peripheral blood lymphocytes during this study.

Overall, the present study describes the dynamics of HIV-1 plasma viremia after removal of virions. In previous studies [5, 6], HIV-1 turnover was analyzed in vivo using specific anti–HIV-1 compounds. Indeed, perturbation of HIV-1 viremia levels with TPEx represents a more direct procedure for the analysis of viral dynamics than does examination of the effect of antiretroviral therapy, since the latter requires absorption and cellular processing of drug to be effective. While the results shown here are in agreement with previous evidence [1, 5–7] indicating that the turnover of circulating virions is a highly dynamic process in HIV-1 infection, they also extend the analysis of HIV-1 dynamics, using a different approach, and indicate that a large proportion of cell-free virions is present in extravascular fluids during HIV-1 infection and tends to be in balance with plasma viremia at any time point.

References

Human Immunodeficiency Virus Type 1 Dynamics in Different Lymphoid Tissue Compartments

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Human immunodeficiency virus type 1 (HIV-1) RNA was measured in total lymph node (LN) tissue and isolated LN mononuclear cells (LNMC) in sequential LN biopsy samples from 1 patient with primary HIV-1 infection and from 5 previously untreated patients with chronic disease. HIV-1 RNA levels were an average of 210-fold higher in total LN tissue compared with levels in LNMC, even during primary infection, when circulating antibodies were absent. After the patients were treated with a three- or four-drug regimen, total HIV-1 RNA decreased exponentially in total LN tissue and in LNMC (mean half-lives of 8.5 ± 1.8 and 7.9 ± 2.2 days, respectively). In addition, the evolution of the infectious virus in LNMC was analyzed for the 5 patients with chronic disease: Titers decreased, with a mean half-life of 7.5 ± 2.3 days. Extracellular virions are the most important virus compartments in LNs; however, they exhibit the same dynamics as virions situated in LNMC, with a mean virus decay half-life of ~1 week.

In the past few years, high levels of human immunodeficiency virus type 1 (HIV-1) turnover have been demonstrated in blood [1–3], and lymph nodes (LNs) have been characterized as the major virus reservoir [4, 5]. Because it remains difficult to obtain sequential LN biopsies in clinical practice, little is known about the dynamics of HIV-1 in these organs. Using the same approach used for studying viral kinetics in plasma, with an antiretroviral combination to perturb the steady state [1, 2], we previously found in 4 patients that the half-life of HIV-1 RNA decrease was 6.01 ± 3.44 days [6]. Those patients were receiving a combination of zidovudine, didanosine, and lamivudine and had four LN biopsies (done with a large needle; one just before treatment and three in the 3 weeks following initiation of treatment).

HIV-1 is present not only in LN mononuclear cells (LNMC) but also as immune complexes at the surface of the dendritic cells [4, 5, 7]. To further characterize the evolution of HIV-1 RNA in total LN tissue and in isolated LNMC, we determined levels of HIV-1 RNA over time in a group of 6 patients. Surgical LN biopsies were done over the 4 months following the initiation of combined antiretroviral therapy.

**Patients and Methods**

**Population.** A 30-year-old woman with primary HIV-1 infection was treated with a combination of zidovudine (200 mg three times a day), didanosine (200 mg twice a day), and lamivudine (150 mg two times a day). In the first 3 months after beginning therapy, she had three LN biopsies. In addition, 5 treatment-naïve patients (mean age, 32 ± 6 years) with chronic HIV disease and CD4+ T cell counts of 50–500 x 10^6/L received a combination of zidovudine (200 mg three times a day), didanosine (200 mg two times a day), lamivudine (150 mg two times a day), and saquinavir (600 mg three times a day). Ketoconazole (200 mg two times a day) was systematically added to this regimen to increase blood levels of saquinavir.

**LN biopsies.** Surgical biopsy of a superficial LN was done under local anaesthetic before therapy and was repeated 2–4 times in the following 4 months.

**LN HIV-1 burden.** One-third of each LN biopsy sample was used to measure total tissue HIV-1 RNA as previously described [6]. In brief, total LN tissue was lysed by incubation with RNA-B (Bioprobe Systems, Montreuil sous Bois, France), which should extract all HIV-1 RNA. The RNA was diluted 1/100, and the liquid phase was used as plasma sample in a polymerase chain reaction (Amplicor HIV-1 Monitor; Roche Diagnostic Systems, Neuilly sur Seine, France). The absence of contaminating DNA was checked by polymerase chain reaction without performing a reverse transcription. The results were expressed in relation to cell number, as calculated by measuring the optic density of LN genomic DNA.
The number of cells was inferred from the total amount of genomic DNA, using a standard curve established with known numbers of peripheral blood mononuclear cells. Two-thirds of each LN sample was cut with a scalpel, and the cells were teased out in RPMI 1640 and subjected to gradient centrifugation using lymphocyte separation medium (Eurobio, Les Ulis, France). Some of the LNMC were cocultivated with peripheral blood mononuclear cells from healthy donors to measure the infectious HIV-1 titer [8, 9]. Others were analyzed for LNMC HIV-1 RNA levels. RNA was extracted with RNA-B from a pellet of 10⁶ cells, and the Amplicor HIV-1 Monitor (Roche) was used for quantitation.

**Statistical analysis.** The evolution of HIV-1 infectious levels and of HIV-1 RNA levels was analyzed by use of linear regression.

**Results**

The patient with primary HIV-1 infection was seronegative by third generation ELISA (Abbott, Abbott Park, IL) and Western blot (HIV blot 2.2; Diagnostic Biotechnology, Singapore) at admission. However, the initial diagnosis of HIV-1 infection was highly probable because the patient had had recent unprotected sex with an HIV-infected man, and she presented with a typical clinical picture of HIV-1 infection. Consequently, an assay for plasma viremia was done immediately, and HIV-1 RNA was present at 1.2 × 10⁶ copies/mL. The first LN biopsy on this patient was done the same day. The level of total LN HIV-1 RNA was 26 × 10⁶ copies/10⁶ cells, and the level of LNMC HIV-1 RNA was 170,000 copies/10⁶ cells. The patient began combination therapy with zidovudine, didanosine, and lamivudine, and 1 month later, her plasma level of HIV-1 RNA was 230 copies/mL, her total LN HIV-1 RNA level was 37,000 copies/10⁶ cells, and her LNMC HIV-1 RNA level was 1250 copies/10⁶ cells. After 3 months of therapy, only a very small superficial LN could be obtained from this patient, and plasma HIV-1 RNA was <20 copies/mL, as determined by use of a boosted assay [10]. Only total LN HIV-1 RNA could be measured in this small LN, and it was 18,000 copies/10⁶ cells.

The 5 patients with chronic disease had a mean level of HIV-1 RNA in total LN tissue that was an average of 210-fold higher (range, 120–230) than that in LNMC before therapy. HIV-1 RNA decreased exponentially in total LN tissue (mean half-life, 8.5 ± 1.8 days) and in LNMC (mean half-life, 7.9 ± 2.2 days), with no statistical difference between these 2 half-lives, after introduction of the four-drug regimen (figure 1). Sufficient LNMC were available in each case to measure infectious titers, and the mean half-life of infectious virus decay was 7.5 ± 2.3 days (figure 2).

**Discussion**

Haase et al. [11] recently measured HIV-1 RNA in lymphoid tissue cells by use of image analysis and compared the level to that of HIV-1 RNA in virions at the surface of the dendritic cells. They found that extracellular virus burden is much greater than the virus burden in lymphoid cells, but the design of their study did not allow assessment of the impact of combined therapy in lymphoid tissue during follow-up. We have studied these aspects, using different methodology, in 6 patients with HIV-1 infection.

Our data confirm that the pool of extracellular HIV-1 RNA is far greater than that of LNMC HIV-1 RNA and that extracellular RNA can accumulate in this compartment very early, even in the absence of circulating antibodies. The effect of antiretroviral therapy on LN virus load has been observed [12–
Measuring the exact effect of therapy on the lymphoid compartment necessitates a longitudinal study before and after the initiation of drugs. In the present study, using different methods to quantify HIV-1 in surgically biopsied LNs, we confirmed our previous findings that the half-life of virus decrease in LNs is $\sim 1$ week.

The methodology for HIV-1 quantitation in lymphoid organs has not yet been the subject of consensus guidelines. Expression of results related to tissue weight, as done by some authors using the branched DNA technique [15], is inadequate because the structure (number of cells and fat infiltration) varies from 1 LN to another. A major advantage of our technique is the use of the number of cells as the denominator.

At a time when most antiretroviral combination therapies seem capable of keeping plasma RNA at undetectable levels, measurement of the residual HIV-1 activity in LNs is the next step in the virologic monitoring of infected patients.

Reference

The Effect of Tenidap on Cytokines, Acute-Phase Proteins, and Virus Load in Human Immunodeficiency Virus (HIV)–Infected Patients: Correlation between Plasma HIV-1 RNA and Proinflammatory Cytokine Levels

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Proinflammatory cytokines may be important in the pathogenesis of human immunodeficiency virus type 1 (HIV-1) disease. Tenidap decreases interleukin (IL)-6, IL-1, and tumor necrosis factor (TNF) production by peripheral blood mononuclear cells and decreases IL-6 plasma levels in rheumatoid arthritis patients. In this randomized double-blind study, 43 HIV-1–infected patients received tenidap (120 mg) or placebo daily for 6 weeks and then crossed over to the alternative therapy for an additional 6 weeks. Mean entry CD4 cell count was 140/μL. Analyses were performed on cytokines, acute-phase proteins, virus load, and CD4 cell counts. With the exception of small differences in plasma TNF levels, tenidap had no significant effect on these indices. Significant correlations of plasma IL-6 and TNF levels with HIV-1 RNA were noted. Six patients discontinued tenidap due to rash. The effects of tenidap in HIV-1 infection contrast to results in arthritis patients, in whom tenidap decreased plasma levels of IL-6 and acute-phase proteins.

Tenidap is a novel antirheumatic antiinflammatory drug that not only inhibits cyclooxygenase but also blocks production of interleukin (IL)-6 in vitro and in vivo [1–3]. The rationale for studying the role of tenidap in human immunodeficiency virus type 1 (HIV-1)–infected patients included the elevated levels of IL-6 in HIV-1–infected persons [4], the ability of IL-6 to induce HIV-1 expression [5], the ability of tenidap to decrease IL-6 both in vitro [2] and in rheumatoid arthritis patients [3, 6, 7], and the ability of tenidap to inhibit HIV-1 replication in infected cell lines [8].

Tenidap inhibits production of IL-6, tumor necrosis factor (TNF), and to a lesser extent IL-1β by human peripheral blood mononuclear cells (PBMC) stimulated with lipopolysaccharide (LPS) [2]. In rheumatoid arthritis patients, tenidap therapy decreases plasma IL-6 over a period of 6–12 weeks, whereas the nonsteroidal antiinflammatory drugs (NSAIDs) piroxicam and naproxen do not [3, 6, 7]. Tenidap also affects intracellular homeostasis. Tenidap inhibits the chloride-bicarbonate exchanger and causes a rapid and sustained acidification of the cytoplasmic compartment [9]. Finally, tenidap, like NSAIDs, inhibits the cyclooxygenase-mediated pathway of arachidonic acid metabolism, thereby decreasing prostaglandin biosynthesis [10].

Tenidap decreases HIV-1 replication in acutely infected lymphocyte and monocyte lines [8]. These effects are seen at concentrations well below those toxic to cells. The antiviral effects of tenidap are mimicked by the anion transport inhibitor UK5099 but not by the NSAID piroxicam [8]. Moreover, the inhibition of HIV-1 production is not reversed by exogenous IL-6, suggesting that the antiviral effects may be independent of the effects of the drug on IL-6 production or cyclooxygenase activity. The antiviral effects of tenidap may be related to its ability to modulate intracellular pH or inhibit the chloride-bicarbonate exchanger.

This randomized placebo-controlled double-blind crossover study was designed to ascertain if tenidap treatment decreases levels of cytokines, acute-phase proteins, or plasma HIV RNA in HIV-1–infected patients. The dose of tenidap used and the length of this study were chosen to match those of a clinical trial of tenidap in rheumatoid arthritis patients in which cytokine modulation by tenidap correlated with clinical parameters.

Materials and Methods

Patient characteristics and treatment plan. Forty-three HIV-1–infected patients with CD4 cell counts ≤400/μL were enrolled between January 1994 and January 1995 at Beth Israel Hospital and Case Western Reserve’s University Hospitals. Patients had absolute neutrophil counts >1000/mm³, platelets >75,000/mm³, alanine and aspartate aminotransferases <3 times the upper limit
of normal, and creatinine clearance >50 mL/min; were receiving prophylactic therapy for Pneumocystis carinii pneumonia (PCP) if their CD4 cell count was <200/mm³, and were willing to discontinue any NSAID therapy. Patients receiving antiretrovirals were on stable regimens for 4 weeks before study entry; antiretrovirals were continued throughout the trial. Exclusion criteria included treatment with corticosteroids, megestrol acetate, or anticoagulants within 1 month of enrollment or presence of active opportunistic infection, chronic inflammatory conditions, neoplastic disease requiring systemic therapy, or peptic ulcer disease. In this double-blind study, each subject was randomly assigned to receive tenidap (120 mg) or placebo daily for 6 weeks; each then crossed over to receive 6 weeks of the other therapy.

Schedule of evaluations. Patients were seen at screening and baseline visits and then at days 0, 7, 21, 42 (crossover day), 49, 63, and 84. Complete blood count, serum electrolytes, blood urea nitrogen/creatinine, liver function tests, glucose, urinalysis, and stool for occult blood were examined at each visit, as were plasma IL-1β, IL-6, and TNF and serum amyloid-A (SAA) and C-reactive protein (CRP). CD4 cell counts and viral studies (p24 and plasma HIV RNA) were obtained at the prebaseline visit and on days 0, 21, 42, 63, and 84. PBMC obtained on days 0, 42, and 84 were tested for cytokine production after in vitro stimulation with LPS.

Laboratory assays. PBMC were isolated by ficoll-hypaque centrifugation at 4°C to prevent activation of cytokine transcription. PBMC were suspended in pyrogen-free RPMI 1640 (Bio-Whittaker, Walkersville, MD) supplemented with 10% fetal calf serum (Hyclone, Logan, UT). supernatants were spun at 70°C. Supernatants levels of IL-6, IL-1β, and TNF-α were measured by ELISA (R&D Systems, Minneapolis). For measurements of plasma cytokines, whole blood was spun at 4°C; plasma was saved and stored at −70°C. Plasma levels of IL-6, IL-1β, and TNF-α were measured by high-sensitivity ELISA (R&D Systems for IL-6 and IL-1β; MedGenix, Brussels, for TNF-α), serum levels of SAA and CRP by ELISA (Hemagen Diagnostic, Waltham, MA); p24 antigen levels after immune complex dissociation (ICD-p24) by ELISA (Coulter Diagnostics, Miami); and HIV-1 plasma RNA levels by the branched DNA (bDNA) assay (Chiron, Emeryville, CA), which has a lower limit of detection of 10,000 RNA copies/mL.

Statistical analysis. Analyses of covariance appropriate for a two-period, two-treatment crossover study, using baseline values as covariate, were performed separately for plasma cytokines (IL-6, IL-1β, TNF-α), inducible cytokines (IL-6, IL-1β, TNF-α), acute-phase proteins (CRP, SAA), and virus load (p24 antigen and HIV-1 RNA). The treatment effect was analyzed on the basis of the natural log-transformed observations obtained at the end of each treatment period for all data except CD4 cell counts, for which untransformed data were used. One was added to every value before the natural log transformation to avoid values equal to zero. Plasma RNA levels <10,000 copies/mL were assigned a value of 5000. For the correlative analyses between plasma cytokine and plasma HIV RNA levels, all available data points were used irrespective of treatment. All significance tests were two-sided. It was assumed that the disease status of the patients did not change over the 12-week trial period and that the 6-week treatment time for each period was long enough both to show activity of the treatment and to wash out any carryover effect from the previous period. There were no statistical carryover effects for all the plasma cytokines, acute-phase proteins, inducible cytokines, and virus load.

Results

Clinical events. Forty-three patients were enrolled. Twelve patients experienced rashes; all occurred while patients were

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**Table 1. Arithmetic mean of cytokine levels, acute-phase proteins, and virus load after 6 weeks of tenidap or placebo treatment.**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Placebo*</th>
<th>Tenidap†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma cytokines (pg/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>40.2</td>
<td>39.6</td>
<td>46.5†</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.1</td>
<td>1.8</td>
<td>2.1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.28</td>
<td>0.30</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Inducible cytokine (pg/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>3468</td>
<td>4078</td>
<td>4818</td>
</tr>
<tr>
<td>IL-6</td>
<td>102,593</td>
<td>106,478</td>
<td>119,012</td>
</tr>
<tr>
<td>IL-1β</td>
<td>4898</td>
<td>5874</td>
<td>5890</td>
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<tr>
<td><strong>Acute-phase proteins (µg/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>4.6</td>
<td>4.5</td>
<td>2.9</td>
</tr>
<tr>
<td>SAA</td>
<td>5.7</td>
<td>6.5</td>
<td>4.9</td>
</tr>
<tr>
<td>CD4 cells/µL, mean (range)</td>
<td>150 (0–461)</td>
<td>166 (0–416)</td>
<td>144 (0–452)</td>
</tr>
<tr>
<td>Viral assays</td>
<td></td>
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<tr>
<td>p24 antigen (pg/mL)</td>
<td>46.4</td>
<td>49.8</td>
<td>47.1</td>
</tr>
<tr>
<td>HIV RNA (Eq/mL)</td>
<td>117,000</td>
<td>125,000</td>
<td>135,000</td>
</tr>
</tbody>
</table>

NOTE. After 6 weeks, placebo recipients were given tenidap and tenidap recipients were given placebo for another 6 weeks. TNF, tumor necrosis factor; IL, interleukin; CRP, C-reactive protein; SAA, serum amyloid-A.

* Days 42 and 84, respectively, for placebo-tenidap and tenidap-placebo sequences.
† Days 42 and 84, respectively, for tenidap-placebo and placebo-tenidap sequences.
‡ P = .003 for plasma TNF levels after 6 weeks of tenidap vs. placebo after being adjusted for baseline. No other comparisons were statistically significant.
receiving tenidap at a median (range) of 11 (9–16) days after beginning tenidap (day 0 for tenidap-placebo sequence, day 42 for placebo-tenidap sequence). Six of these patients discontinued the study drug. Nine other patients also discontinued the study drug: 5 who were receiving tenidap (3 for noncompliance, 1 for grade IV anemia, 1 for urinary tract infection) and 4 who were receiving placebo (2 for pneumonia [E. coli, PCP], 1 for Cryptosporidium and Campylobacter diarrhea, 1 for cryptococcal meningitis). Except for rash, no other adverse event was attributed to study drug.

**Cytokines, acute-phase proteins, virus load, and CD4 cell counts.** The only treatment effect observed was a small but significant increase in plasma TNF during treatment with tenidap (table 1). Since only 4 of 28 patients were not taking antiretroviral agents at baseline, it was not feasible to perform separate analyses for those taking antiretrovirals versus those who were not.

The analysis of the effect of tenidap on cytokines, acute-phase proteins, and virus load is a subgroup analysis based on the 28 patients who completed the 12-week study. Since this subgroup may not be representative of the entire study population, a potential bias must be kept in mind. Patients who completed the study were comparable to the dropouts in terms of age (mean of all patients, 38.8 years), known duration of infection (mean of all patients, 4.0 years), and use of antiretroviral agents at baseline (88% of all patients). The mean (range) baseline CD4 cell count of all patients was 140 (0–461), of completers was 150 (0–461), and of dropouts was 119 (0–364) cells/μL.

To test the relationship between levels of plasma cytokines and plasma HIV RNA, correlative analyses were performed. Both plasma IL-6 and TNF levels correlated with HIV RNA (P = .0002 and .004, respectively), whereas plasma levels of IL-1β did not (P = .58) (figure 1).

**Discussion**

A 6-week trial of tenidap therapy had no demonstrable effects on levels of the cytokines IL-1β and IL-6, on CD4 cell counts, on acute-phase proteins (SAA, CRP), or on measurements of plasma virus load (p24, HIV-1 RNA) in this double-blind crossover trial conducted among patients with moderately advanced HIV-1 disease.

Proinflammatory cytokine expression may be increased in AIDS patients. Elevated levels of serum or plasma cytokines and increased production of cytokines by cultured PBMC have been observed; however, these findings have not been consistently reproduced (reviewed in [11]). In vitro, IL-6 induces HIV expression alone and synergistically with TNF [5]. The present clinical trial permitted us to explore whether the in vitro correlation between cytokines and HIV-1 expression has a parallel in an in vivo setting. In this study, plasma levels of IL-6 and TNF were partly correlated with plasma HIV RNA, though levels of IL-1β were not. It is not clear whether these correlations represent the effects of cytokines on HIV-1 replication or vice versa (or both).

The only treatment effect observed in this study was a small but significant increase in plasma TNF levels after 6 weeks of tenidap therapy. The mean difference in TNF levels was 6.9 pg/mL, ~17% of baseline values. This effect may be related to the ability of tenidap to decrease prostaglandin synthesis.
Prostaglandin E2 is known to down-regulate TNF gene expression in mouse peritoneal macrophages [12]. Similar increases in TNF might be expected with other prostaglandin synthesis inhibitors, such as ibuprofen and indomethacin. While TNF-α activates expression of HIV-1 in vitro through activation of the transcription factor NF-κB [13], the effect of tenidap on TNF in this study was not associated with an increase in HIV-1 RNA.

The patients in this study had a mean (range) baseline CD4 cell count of 140 (0–461) cells/μL; 88% were receiving antiretroviral therapy. Virus load is a useful partial surrogate marker of HIV-1 disease progression with advantages over CD4 T lymphocyte counts [14]. The bDNA assay for plasma HIV-1 RNA provides a good measure of virus burden [14]. As measured by this assay and by ICD p24 levels, no treatment effect of tenidap was seen.


Tenidap was well tolerated except for generalized maculopapular rashes that occurred ~11 days after start of treatment in 12 (28%) of 43 patients. Six of these patients discontinued the study drug, making a morbilliform rash the adverse event that lead to the greatest number of discontinuances. Such adverse cutaneous drug reactions have been recognized frequently in HIV-infected individuals and have been associated most prominently with trimethoprim-sulfamethoxazole [15]. Of note, rashes were not seen frequently in tenidap-treated arthritis patients [3]. No other adverse events were attributed to the study drug.

The low levels of plasma IL-6 in this study may have precluded demonstration of an effect of tenidap on this cytokine. Circulating levels of IL-6 in persons with HIV-1 infection and AIDS vary according to patient populations and the assays used. Using a high-sensitivity ELISA kit, we found plasma IL-6 levels ranging from 8 to 104 pg/mL in AIDS patients with opportunistic infections (unpublished data) and from 0.2 to 11 pg/mL (arithmetic mean, 2.1) in healthy HIV-infected patients in this study. Using an earlier version of this assay, Littman et al. [3] found mean IL-6 plasma levels of 14 pg/mL in patients with active rheumatoid arthritis. Using a bioassay, others had found mean plasma levels of IL-6 of 120 pg/mL in healthy HIV-infected subjects [4]. Although it is conceivable that an effect of tenidap on plasma IL-6 might have been seen if we had used a bioassay, given the general lack of effect on other cytokines (plasma and inducible), acute-phase proteins, and virus load, future trials of tenidap in HIV-1–infected patients are probably not warranted.

Acknowledgments

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References

High-Dose Interferon-α2a Exerts Potent Activity against Human Immunodeficiency Virus Type 1 Not Associated with Antitumor Activity in Subjects with Kaposi’s Sarcoma

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Anti–human immunodeficiency virus type 1 (HIV-1) activity was assessed in HIV-1–infected homosexual and bisexual men receiving 18–36 MIU/day of recombinant interferon (IFN)-α2a for Kaposi’s sarcoma (KS). The median baseline HIV-1 RNA level was 4.99 log$_{10}$ copies/mL. Seventeen subjects (68%) showed an RNA decline $\geq$5 log$_{10}$/mL, with a maximum at week 4 (median decline = 1.91, range = 3.64–1.15; $P = .0007$), after which RNA levels stabilized. Eight subjects (32%) with lower median initial CD4$^+$ T cell counts (60 vs. 350 × 10$^3$/L; $P = .01$) did not show RNA responses. Neither RNA nor KS responses were negatively affected by IFN-α2a dose modifications.

Anti-HIV responses of KS responders ($n = 15$) and nonresponders ($n = 10$) did not differ. High-dose IFN-α can exert potent anti-HIV activity that is not associated with anti-KS activity.

Interferon (IFN)-α is a cytokine that is mainly produced by peripheral blood mononuclear cells after stimulation with various viral and nonviral stimuli. It is involved in the regulation of many cellular functions, resulting in antiviral, antitumor, and immunomodulatory activities [1]. Soon after it was discovered that human immunodeficiency virus (HIV) causes AIDS, the antiretroviral capacity of IFN-α was demonstrated in vitro [2, 3]. The mechanisms of action of IFN-α in HIV infection are not fully elucidated. An important inhibitory action of IFN-α on HIV replication is interference with assembly and budding of new HIV virions [4].

Clinical results with IFN-α for the treatment of HIV infection have been discouraging. After the demonstration in vitro of synergistic antiviral activity with IFN-α plus zidovudine [5], combinations of low-dose INF-α and zidovudine have been used in different clinical settings with different primary end points and viral parameters [6–9]. However, the largest of these studies showed no evidence of superior clinical benefit from combination therapy: It compared, in a randomized and controlled fashion, a combination of low-dose INF-α and zidovudine with zidovudine monotherapy in treatment-naive HIV-infected subjects [8]. These disappointing clinical results might be due to the use of low-dose IFN-α, because high-dose IFN-α monotherapy for the treatment of HIV-related Kaposi’s sarcoma (KS) has been reported to suppress HIV-1 p24 antigen and provide clinical benefit [10, 11].

Therefore, we conducted a study to explore the anti-HIV activity of high-dose recombinant IFN-α2a, using serum HIV-1 RNA quantification as the most reliable and exact viral parameter. We used stored serum samples from subjects who had participated in a KS treatment study with high-dose recombinant IFN-α monotherapy (27–36 MIU/day) in 1988; all had demonstrated clear antitumor activity and an anti-HIV response [10]. In addition, that study found evidence of an association between antitumor and anti-p24 responses, which was later confirmed by others [11].

Materials and Methods

Study population and design. Twenty-five HIV-1 seropositive homosexual or bisexual men with histologically confirmed and progressive KS showing measurable lesions were included. Additional inclusion criteria are described in detail elsewhere [10]. Clinical and laboratory assessments were conducted at four weekly intervals. The major KS response evaluation was performed at week 8. Subjects with a complete or a partial KS response or with stable KS disease were called KS responders; subjects with progressive disease were called KS nonresponders. Stable disease was categorized as a KS response because an important inclusion criterion was the presence of progressive KS (i.e., new or growing lesions). KS responses were defined according to guidelines described in detail elsewhere [10]. Subjects could not have received any previous treatment for HIV infection and were not allowed to use any known antiretrovirals or immunomodulatory agents during the study. For the purpose of the current study, serum samples up to 12 weeks after initiation of IFN-α2a therapy were available for...
quantitation of HIV RNA: Patients received IFN-α2a daily during the first 8 weeks; during the subsequent 4 weeks, regimens were modified according to the KS response at week 8. An HIV RNA response was defined as a decrease from baseline of at least .5 log₁₀ copies/mL at two consecutive time points (or for at least 1 month).

**Intervention.** The IFN-α used in this study was recombinant human IFN-α2a (Roferon; Roche, Basel, Switzerland) and was self-administered by subcutaneous injection. For days 1–5, subjects received doses that increased daily by stepwise increments (i.e., 3, 9, 18, 27, and 36 MIU, respectively). Thereafter, for the first 8 weeks, subjects received 36 MIU/day if their body weight was ≥60 kg and 27 MIU if it was <60 kg. After 8 weeks, IFN-α2a was administered every other day to patients with a partial KS response or stable disease. For those with a complete KS response or progressive disease, treatment was discontinued.

**HIV-1 RNA quantification.** Serum was stored at −70°C. Serum HIV-1 RNA was quantitated in once-thawed serum, using nucleic acid–based sequence amplification (Organon Teknika, Boxtel, Netherlands) [12]. The variation of quantitative results is within .5 log₁₀ copies/mL. The lower limit at which RNA can be reliably quantified is 1000 copies/mL (3 log₁₀ copies/mL).

**T cell subpopulations and HIV-1 p24 antigen.** Peripheral blood CD4⁺ and CD8⁺ T lymphocyte subpopulations were enumerated using dual-color immunofluorescence and flow cytometry. Serum HIV-1 p24 antigen was measured by a sandwich-type ELISA (Abbott Laboratories, Abbott Park, IL).

**Statistics.** Laboratory values for CD4⁺ cells and RNA were not normally distributed, partially due to censoring of the data (RNA). HIV-1 RNA results were log-transformed, and values below the lower quantification limit were considered to contain 3 log₁₀ RNA copies/mL. Baseline laboratory values are the means of two pretreatment values (one assessment within 4–8 weeks before entry and one at the start of treatment). Fifteen subjects (60%) had only one pretreatment RNA value available. Comparisons between groups were conducted at weeks 0, 4, and 8, using the Mann-Whitney U test. The HIV RNA response over time in the total study population was tested by the Wilcoxon signed rank test for paired observations. Comparisons of the proportion of either HIV-1 RNA or KS responders and nonresponders stratified by CD4⁺ cell counts below or above 200 × 10⁶/L were tested by the χ² test. The correlation between serum HIV-1 RNA and p24 antigen concentrations was tested by Spearman’s rank correlation coefficient. All tests were two-sided, and significance was set at P < .05.

**Results**

**Study population.** At baseline, the median CD4⁺ cell count was 280 × 10⁶/L (range = 10–670 × 10⁶), and the median serum HIV-1 RNA concentration was 4.99 log₁₀ copies/mL. Three subjects with only one baseline serum sample available had HIV-1 RNA concentrations below the lower quantification limit of the RNA assay. In the 14 subjects (56%) with p24 antigenemia (≥30 pg/mL), no correlation between serum HIV-1 RNA and p24 antigen concentrations was found at baseline or at any analyzed time point thereafter (data not shown).

**HIV-1 RNA response.** In all subjects, the maximum change in serum HIV-1 RNA concentrations during IFN-α2a treatment was observed at week 4 (median = −.79 log₁₀/mL, range = −3.64 to +2.62; P = .001). However, this group contained RNA responders (n = 17; 68%) as well as nonresponders (n = 8; 32%). Seventeen HIV RNA responders showed RNA declines of ≥1 log₁₀ copies/mL, whereas the 8 RNA nonresponders showed clear increases relative to baseline (median RNA increase = 1.3 and 1.5 log₁₀/mL at weeks 4 and 8, respectively). The RNA responders showed a median RNA decrease from baseline of 1.91 log₁₀/mL (P = .0007) at week 4 and 1.78 log₁₀/mL (P = .005) at week 8 (figure 1). For 5 subjects (20%) at week 4 and 4 subjects (21%) at week 8 after the start of IFN-α2a therapy, serum HIV-1 RNA levels were below the quantification limit of the assay (3 log₁₀/mL).

Comparison of the baseline characteristics of the HIV RNA responders and nonresponders revealed that RNA nonresponders had significantly lower median CD4⁺ T cell numbers than did the responders (60 vs. 350 × 10⁶/L; P = .01). Five of the RNA responders (29%) versus 6 of the RNA nonresponders (75%) had baseline CD4⁺ cell counts of <200 × 10⁶/L, whereas 12 RNA responders (71%) versus 2 nonresponders (25%) had baseline CD4⁺ cell counts of ≥200 × 10⁶/L (P = .032). Other baseline variables, such as age, p24 antigenemia, KS response, and RNA concentrations did not show differences (data not shown). During IFN-α2a therapy, CD4⁺ cell responses (percentages and numbers) relative to baseline responses did not show different patterns between patient response groups at any analyzed time point (data not shown). The RNA nonresponder group included 2 of 3 subjects with censored baseline RNA levels of 3 log₁₀ copies/mL whose RNA concentrations became detectable during therapy.

**KS response.** Fifteen subjects (60%) showed a KS response, whereas 10 subjects (40%) showed progressive KS disease. At baseline, mean age (35.2 vs. 39.7 years) and median baseline CD4⁺ cell counts (230 vs. 350 × 10⁶/L) were lower.
in the group of KS nonresponders than in the responders, but the difference was not significant. All subjects with HIV-1 RNA concentrations below the assay quantification limit at any time point belonged to the group of KS responders. RNA concentrations did not show different patterns between KS responders and nonresponders during the first 8 weeks of daily IFN-α2a administration: at week 4, the median RNA concentration relative to baseline was $-1.91 \log_{10}/mL$ ($-3.64$ to $+2.62$) versus $-1.47 \log_{10}/mL$ ($-2.28$ to $+1.9$); at week 8, the median RNA concentration relative to baseline was $-1.41 \log_{10}/mL$ ($-3.64$ to $+2.33$) versus $-1.48 \log_{10}/mL$ ($-1.92$ to $+1.65$), respectively.

Within the group of KS responders, RNA responses of subjects with either complete ($n = 2$) or partial response ($n = 8$) or with stable disease ($n = 5$) were indistinguishable by RNA response (data not shown). At weeks 4 and 8, median CD4+ T cell counts relative to baseline were higher in the KS responders than in the nonresponders, although these differences did not reach significance ($+10$ vs. $-40 \times 10^6/L$; $P = .09$ at week 4; $0$ vs. $-40 \times 10^6/L$; $P = .14$ at week 8). Concomitant KS and RNA responses were found in 10 subjects (40%), whereas a combined KS and RNA nonresponse was observed in 3 (12%). All other subjects ($n = 12$; 48%) had divergent KS and RNA responses (table 1).

**Dose-response relation.** HIV RNA and KS responses in relation to the dose of IFN-α2a are listed in table 1. Full-dose therapy was considered to be treatment with 36 MIU IFN-α2a daily, whereas all other dosages were considered to be a reduced IFN-α2a dose. Five subjects (20%) had dose modifications before week 8. Of them, 4 had toxicity-related dose reductions to 18 MIU daily and 1 had a short-lasting treatment interruption. Table 1 shows that neither the HIV RNA response nor the KS response seems to be affected by a reduced IFN-α2a dose.

After the KS response evaluation at week 8, treatment was stopped for 12 subjects because of progressive KS ($n = 8$) or personal request, mainly because of side effects ($n = 4$). Thirteen subjects (52%), including 2 with progressive KS (protocol violation), continued to use IFN-α2a every other day. Twelve of these subjects had serum samples available at week 12. Between week 8 and 12, RNA increased ($>5 \log_{10}/mL$) in 4 subjects (33%), including both subjects with progressive KS, whereas RNA declined ($>5 \log_{10}/mL$) in 2 subjects (17%). In the other subjects ($n = 6$; 50%), changes in RNA levels were within $5 \log_{10}/mL$.

**Discussion**

In the present study, high-dose recombinant IFN-α2a exerted potent anti-HIV activity in a substantial subgroup of subjects treated with the agent for AIDS-related KS. A previous study established the anti-HIV activity of IFN-α alone, as measured by serum HIV-1 RNA concentrations [13]. However, direct comparison of the two studies is hampered by differences in study designs, IFN-α preparations (natural human IFN-α or IFN-α3, consisting of a mixture of different IFN-α species), and clinical parameters of the study populations (disease stage, CD4+ cell counts, and HIV-1 RNA levels). Despite the present results, it should be emphasized that IFN-α2a exhibited no anti-HIV response in 32% of our subjects. This group of nonresponders might be slightly greater, considering that no serum samples were available at week 8 for 6 subjects. However, these subjects were considered to be RNA responders because of unequivocal RNA declines at week 4. In addition, only 1 of 8 RNA nonresponders showed a biphasic RNA response.

Although the number of RNA nonresponders is small, the increase of HIV-1 RNA levels in some of these subjects could not be explained by noncompliance or intercurrent infections. The only significant difference between the RNA responders and nonresponders was the lower initial CD4+ cell counts in the latter group. The KS nonresponders also had lower baseline CD4+ cell counts than KS nonresponders; however the difference was not significant, probably due to the small sample size. In various studies, CD4+ cell counts $>200 \times 10^6/L$ have been associated with favorable KS responses to IFN-α [10, 11] or suppression of serum HIV p24 antigen levels (or both) [11, 14]. Adequate anti-HIV and anti-KS activities of IFN-α appear to require a certain minimum level of cellular immunity.

To date, the minimal effective dose of IFN-α in HIV infection is still unclear. Within the dose range of the current study ($\geq 18$ MIU/day up to week 8) no dose-effect relationship could be demonstrated, possibly due to the small sample size. One study using a new natural IFN-α at different dose levels (1, 5, and 12.5–20 MIU three times a week) showed a dose-dependent anti-HIV effect [13]. In the highest dose group, 3 of 5 subjects had $\geq 2.41 \log_{10}/mL$ RNA decline, whereas the decline of serum RNA concentrations was $<1 \log_{10}/mL$ with the two lower dose levels. Another study, using HIV p24 antigen as the viral parameter, demonstrated a dose-dependent p24 antigen suppression even with the use of low-dose IFN-α (2–6 MIU/day) [9].

### Table 1. Distribution of 25 study subjects given different doses of IFN-α2a for treatment of Kaposi’s sarcoma (KS), according to size of dose received, HIV RNA response, and KS response.

<table>
<thead>
<tr>
<th>Dose size, KS response</th>
<th>RNA response</th>
<th>No RNA response</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 MIU/day ($n = 18$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KS responder</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>KS nonresponder</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>18–27 MIU/day* ($n = 7$)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>KS responder</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>KS nonresponder</td>
<td>2</td>
<td>1</td>
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</table>

NOTE. Data are no. of subjects. KS responders = subjects with complete or partial KS response or with stable KS disease; KS nonresponders = subjects with progressive disease. HIV RNA response was defined as decrease from baseline of at least $5 \log_{10}$ copies/mL at 2 consecutive time points (or for at least 1 month).

* 27 MIU was weight-adjusted dose (<60 kg).
An important observation in the current study was the lack of an association between the anti-HIV and anti-KS responses: Previous reports on IFN-α therapy in KS had suggested an association between the two responses [10, 11]; however, the anti-HIV response in those studies was assessed by the reduction of serum HIV p24 antigen levels. We and others [15] were unable to demonstrate a correlation between serum HIV p24 antigen and RNA concentrations. The anti-KS activity of IFN-α might be associated with inhibition of KS-associated herpes-like virus (KSHV). Development of viral quantitation techniques for KSHV might elucidate this question.

In conclusion, high daily doses of IFN-α can exert potent antiretroviral activity. Low CD4+ T cell counts seem to impair both the anti-HIV and the anti-KS response. These responses to IFN-α show no association. Despite disappointing previous clinical data from treatment with low-dose IFN-α, it is too early to completely abandon IFN-α for the treatment of HIV-infected subjects with moderate immune impairment. The dose and type of IFN-α with the best ratio between activity and toxicity, either in monotherapy or combination therapy, and the factors interfering with the response to IFN-α remain to be established.

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References

A Prolonged Outbreak of *Escherichia coli* O157:H7 Infections Caused by Commercially Distributed Raw Milk

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A protracted outbreak of *Escherichia coli* O157:H7 infections was caused by consumption of unpasteurized (“raw”) milk sold at Oregon grocery stores. Although it never caused a noticeable increase in reported infections, the outbreak was recognized because of routine follow-up interviews. Six of 16 Portland-area cases reported between December 1992 and April 1993 involved people who drank raw milk from dairy A. By pulsed-field gel electrophoresis (PFGE), *E. coli* O157:H7 isolates from these cases and from the dairy A herd were homologous (initially, 4 of 132 animals were *E. coli* O157:H7-positive). Despite public warnings, new labeling requirements, and increased monitoring of dairy A, retail sales and dairy-associated infections continued until June 1994 (a total of 14 primary cases). Seven distinguishable PFGE patterns in 3 homology groups were identified among patient and dairy herd *E. coli* O157:H7 isolates. Without restrictions on distribution, *E. coli* O157:H7 outbreaks caused by raw milk consumption can continue indefinitely, with infections occurring intermittently and unpredictably.

*Escherichia coli* O157:H7 outbreaks are most commonly caused by consumption of undercooked beef or other foods that are cross-contaminated with beef. Such outbreaks are usually detected because of a marked increase in illness within a group or community. Common-source outbreaks are typically short-lived, limited by the quantity and shelf life of the contaminated product(s), and are preventable with adequate kitchen hygiene and cooking.

While often contaminated with enteric organisms during collection, before sale almost all milk is pasteurized, which kills pathogenic microbes. Some consumers, however, choose to drink unpasteurized milk, despite the well-documented risk of enteric infection [1]. Milk has a limited shelf life, but dairy animals can be productive for years, and the output of a pathogen-colonized dairy herd may be contaminated indefinitely. Thus, depending on product distribution and frequency of contamination, raw milk–associated infections can be widely scattered in both time and place, making it difficult to recognize a common source.

Mandatory reporting of *E. coli* O157:H7 infections in Oregon was initiated in 1990. Reports are first investigated by local health department personnel. During the first few weeks of April 1993, 3 ostensibly unrelated patients in one county mentioned consuming raw milk from a single dairy farm (dairy A). The retail sale of raw milk is legal in Oregon; dairy A milk was sold at supermarkets and other grocery stores in the tricounty Portland area. Here we present the results of our investigation and their implications for disease surveillance and outbreak control.

**Methods**

*Case finding and definitions.* We defined cases as persons from whose stools *E. coli* O157:H7 was cultured or persons who developed diarrhea (≥3 loose stools within a 24-h period) within 7 days of the symptom onset of a culture-confirmed household member. Cases were identified from routine surveillance reports. For this investigation, we reviewed cases with symptom onsets from January 1992 through June 1994 among residents of the three counties (Multnomah, Washington, and Clackamas) comprising greater Portland. When initially reported, cases (or household informants) had been interviewed, using a standardized questionnaire, about possible exposures. We defined raw milk–associated cases as those who reported drinking raw milk within the 10 days before symptom onset. Persons whose illness began ≥2 days after that of another household member were considered secondary cases.

*Statistics.* The probability of finding as many raw milk drinkers as we did among reported cases was estimated using a standard binomial model. We used an arbitrarily chosen interval extending from 1 month before the first raw milk–associated case was reported to the date the cluster was first identified (1 December 1992 to 20 April 1993).
Dairy sampling and microbiology. Agriculture inspectors routinely (every 8 weeks) visit Oregon dairies that sell retail raw milk; milk samples are collected and assayed by standard methods for total coliform count and other parameters. We reviewed milking operations and test results for dairy A from January 1992 through June 1994. Milk samples collected from the bulk tank at least every 2 weeks after April 1993 were assayed for E. coli O157:H7 by two methods: 1 aliquot was cultured directly on sorbitol-MacConkey’s agar; another was enriched overnight and screened by EIA (EHEC-Tek; Organon Teknika; Durham, NC).

Rectal swabs were obtained from all cattle at dairy A and cultured [2]. Bovine isolates and isolates from human cases were confirmed as E. coli O157:H7 by standard biochemical tests, latex agglutination assay, and serologic testing [3].

Isolates were digested with XbaI and subtyped by pulsed-field gel electrophoresis (PFGE) [4]. Relative to one another, PFGE patterns were categorized as indistinguishable, homologous (one- or two-band difference), or unique.

Results

Initial epidemiologic findings. Sixteen primary cases had been reported in the Portland area from 1 December 1992 through 20 April 1993; 6 of these individuals reported drinking raw milk, all from dairy A (figure 1). The first raw milk–associated case became ill in late December 1992. By chance, the probability of finding at least 6 of 16 persons chosen at random to be dairy A milk drinkers is 0.0000000073, assuming that 1% of people drink that brand during a 10-day interval. (Dairy A’s share of the Portland milk market was <0.5%.)

Dairy operation. Dairy A produced ~1400 L of whole milk, skim milk, and cream per day. Milk from the bulk tank was tested 26 times between December 1992 and June 1994, with total coliform levels ranging from undetectable to 17,000 cfu/mL; 5 samples had levels >150 cfu/mL. There was no obvious relationship between total coliform levels and the occurrence of infections. E. coli O157:H7 was never recovered from any dairy A milk sample.

Control measures and subsequent cases. Through media contacts on and after 21 April 1993, we warned the public of the hazards associated with raw milk, particularly from dairy A. Raw milk sales from dairy A were briefly suspended but resumed when E. coli O157:H7 was not recovered from a milk sample or a convenience sample of manure specimens collected at the dairy on 20 April. Legal analysis suggested that Oregon public health agencies lacked clear authority to restrict milk sales unless it could be demonstrated that specific containers destined for sale were contaminated.

Two more dairy A–associated cases were reported in June 1993, 1 with an April onset. In December, the Oregon Department of Agriculture introduced labeling requirements for raw milk products (“This product has not been pasteurized [and] may contain disease-producing organisms”) and new standards for total coliform levels in retail raw milk (<10 cfu/mL). Dairy A agreed to suspend retail sales should additional illnesses be

Figure 1. Reported monthly occurrence of primary E. coli O157:H7 infections in 3 Portland-area counties, January 1992 to June 1994. □ = dairy A–associated E. coli O157:H7 cases; □ = other reported cases. Cases are shown by county of residence and month of symptom onset; within each month, cases are arranged (from bottom to top) in order of onset. Secondary cases (n = 7, including 3 associated with this outbreak) are not shown.
Subtyping of dairy A-associated E. coli O157:H7 isolates from primary cases and dairy herd animals, shown by month of symptom onset and month of culture, respectively. (Only 1 isolate was subtyped from 1 1994 household with 4 co-primary cases.) Homology (≤ 2 band difference by pulsed-field gel electrophoresis) is indicated by circle color; distinguishable patterns are indicated by letters.

Discussion

This outbreak never caused an obvious increase in disease reports from any one county or statewide. It was only recognized because potential exposures, including raw milk consumption, were reviewed during routine follow-up investigations. Such interviews, while labor-intensive, can be essential for identifying protracted and low-intensity outbreaks, particularly when background rates are high.

Suspicions were first voiced when 3 of the April cases in one county reported drinking raw milk—a relatively uncommon exposure. Other raw milk drinkers were then identified from a review of existing surveillance reports. All were confirmed to have consumed milk from dairy A. We then faced the dilemma of when to take public action. The binomial calculation, while using an arbitrarily selected time interval, suggested that a chance association between infection and consumption of dairy A milk was extremely unlikely. Given the ongoing nature of the outbreak, we elected to notify the public immediately rather than delay until a formal study could be done. In the absence of a distinct outbreak period, it was not clear how to delimit a case-control study without significant bias. A cohort study was also infeasible.

The association was eventually corroborated by PFGE subtyping results suggesting that the isolates from milk drinkers had a common origin with each other and with isolates from the dairy herd. Such subtyping can be of great value in outbreak investigations, although as this cluster illustrates, outbreaks are not necessarily marked by a single PFGE pattern.

Anecdotal reports and case-control studies suggest that raw milk is one cause of sporadic E. coli O157:H7 infections [5–8], but only one cluster due to raw milk consumption, an outbreak among Canadian schoolchildren visiting a dairy farm, has been well described [9]. Several other raw milk–associated E. coli O157:H7 clusters in Britain received brief mention [10, 11]. Pasteurized dairy products (yogurt [12] and milk [13]) were implicated in two other British outbreaks, apparently due to contamination with raw milk or improper processing.
Raw milk is an unsurprising vehicle for enteric infections [1], as it unavoidably contains fecal organisms from the milking herd. Occasionally, high levels of contamination can occur as a result of poor environmental sanitation or improper holding temperatures with attendant bacterial replication. Given their prevalence in cattle, these contaminants will sometimes include *Escherichia coli* O157:H7, *Campylobacter* or *Salmonella* species, or other pathogens. Routine inspection of raw milk dairies by governmental agencies cannot prevent this contamination, and indeed may convey a misleading imprimatur of safety to unwary consumers.

While *Escherichia coli* O157:H7 has been isolated from raw milk [11, 14], testing production lots for specific pathogens is an unreliable means of protecting consumers. Contamination may be intermittent or below the detection limit of available assays. Moreover, using a battery of pathogen-specific premarket tests would unrealistically delay distribution and would offer no protection against uncharacterized agents. Assaying for non-specific indicators of fecal contamination would be similarly impractical, and such measures are probably unreliable predictors of risk [15].

Unlike most clusters of foodborne illness, raw milk–associated outbreaks have the potential to persist indefinitely, with cases occurring intermittently and unpredictably. Dairy A–associated infections were scattered over 18 months. Even when a source is identified, these outbreaks can be difficult to control. No known measures will eradicate *Escherichia coli* O157:H7 from individual cattle, much less entire herds, or eliminate fecal contamination of milk as it is collected.

The only effective way to stop raw milk–associated disease is to stop people from drinking raw milk—easier said than done. Despite widespread publicity about the links between dairy A raw milk and potentially life-threatening infection, sales of this brand (and intermittent infections) continued until the dairy was forced out of the retail business. Many raw milk consumers remain skeptical about the inherent hazards of this product or are indifferent to the risks to themselves and their children. Short of an outright ban on sales, which has been enacted in at least 22 states (Klontz KC, personal communication), Canada, and Scotland, continuing consumer education and increasing financial risks for suppliers may be the only means to reduce raw milk consumption and associated illness. In response to this and another outbreak, legislation to outlaw the retail sale of raw milk in Oregon was introduced in 1995. It died in committee.

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References
Utilization and Cost of Serologic Tests for Lyme Disease in Maryland

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To ascertain use of serologic tests for Lyme disease (LD) in Maryland, all laboratories registered with the State Health Department were surveyed. Results show that from 1992 to 1995, 17 laboratories performed 100,000 serologic tests costing $7.1 million on Maryland residents; 90% of these tests were EIAs. The proportion of positive EIAs increased from 3.4% in 1992 to ~7.0% in 1994 and 1995, and the percentage of positive second tests (Western blot, WB) fell from 7.9% to 5.0%–5.5%. The large number of EIAs performed in comparison with the low incidence of LD in the state results in a low predictive value of a positive EIA test. Therefore, the WB is indicated to confirm equivocal and positive EIA tests when characteristic clinical findings of LD are not present. The 30,000 tests for LD performed annually on Maryland residents at a cost of over $2 million in direct medical costs must be added to the public health burden of LD in this state.

Lyme disease (LD) is the most common vectorborne illness in the United States and is increasing in prevalence and distribution [1]. The hallmark of early infection, the expanding bull’s-eye erythema migrans (EM) rash, is absent in 30%–40% of patients meeting the Centers for Disease Control and Prevention’s (CDC) surveillance case definition for LD [2]. Rheumatologic, neurologic, and cardiac manifestations suggestive of late-stage LD require serologic confirmation, since they are often confused with other diagnoses. The association of fibromyalgia and chronic fatigue syndrome with LD is controversial [3]. Therefore, in acute cases in which EM is not present, and in patients with more chronic complaints, serologic testing is relied upon to support the diagnosis.

The most commonly performed test, an EIA, lacks sensitivity in detecting Borrelia burgdorferi antibodies in the earliest stages of the disease. In patients with late-stage LD, cross-reactivity during testing lowers specificity and causes confusion with several infectious and autoimmune diseases [4–6]. The current recommendation is to use a two-test approach to serologic testing for LD: the more sensitive EIA test followed by the more specific Western blot (WB) for those with equivocal and positive EIA test results [4–6].

In 1992, we began collecting and recording data, including diagnostic laboratory tests, on patients with LD reported to the Maryland Department of Health and Mental Hygiene (DHMH). Both data from this LD registry and a physician survey [7] suggested that a large number of diagnostic serologic tests for LD were being performed on Maryland residents. Therefore, we surveyed all laboratories performing diagnostic testing for LD on serum from Maryland residents to estimate the number and types of tests performed and their cost.

Methods

A list of laboratories licensed by the state of Maryland to perform serologic and microbiologic tests was obtained from the DHMH. Each registered laboratory was called and asked if they had performed serologic tests for LD on specimens from Maryland residents from 1 January 1992 through 31 December 1995. Those indicating they performed tests, as well as any laboratory reporting a positive test result to the DHMH, were sent a questionnaire with an explanatory cover letter. The questionnaire inquired about which tests were performed, the number of tests performed per year, the proportion of positive tests, and charges for performing the tests. Each laboratory was assured of the confidentiality of its responses and was assigned an identification number so that results could not be traced to specific laboratories.

The survey was mailed to the 27 laboratories identified. Those that failed to return the survey within 2 weeks were called and reminded. Two weeks later, the remaining laboratories were phoned and offered the option of having a project investigator collect the data from their records. Three chose to be site-visited; test procedures were observed and results were recorded from their records. Three laboratories stated they had not performed LD serology on any samples from Maryland during the 4 years. Five laboratories were involved in mergers, leading to a revised total of 19 laboratories performing LD serology. Two of the 19 laboratories were unable to provide the requested Maryland-specific information, resulting in responses from 17 of the 19 laboratories performing LD serology on Maryland residents. From information provided during the discussions, we estimate these 2 laboratories were each performing <500 serologic tests for LD on Marylanders annually. In addition, several physicians caring for patients with possible LD or tick exposures sent serum samples to nonregistered research laboratories outside the state. These data and those from physicians and laboratories in the LD registry indicated that our sample includes 75%–85% of the serologic tests performed on...
Maryland residents during the study period. The data were analyzed using statistical programs included in Epi Info 6.0 (CDC, Atlanta).

Results

All 17 laboratories used commercial EIA test kits for serologic tests for LD from the following manufacturers: 6, Cambridge Biotech (Worcester, MA); 5, BioWhittaker (Walkersville, MD); 2, MarDx Diagnostics (Carlsbad, CA); and 4, others. The total number of serologic tests for LD increased from 18,144 in 1992 to ~30,000/year in 1994 and 1995 (table 1). The mean commercial laboratory charge for an EIA test was $52 (range, $11–$114), while the average charge for the WB was $106 (range, $92–$128). The average cost of each WB varied little between laboratories. However, the average charge for each EIA was highly variable and was greater than $52, since the laboratory performing the most tests (30,958) charged $114/test. At the other extreme, the Maryland DHMH performed 10,168 EIA tests at no charge and did not perform WBs. The total charges for LD serology doubled from about $1 million to $2 million between 1992 and 1994.

The number of WB tests markedly increased from 1992 to 1993, when the number of laboratories offering this test increased from 1 to 7. Also, the proportion of positive EIA tests doubled from 3.4% in 1992 to 7.0% and 6.9% in 1994 and 1995, respectively. At the same time, the proportion of positive WBs fell from 7.9% in 1992 to 5.0% and 5.5% in 1994 and 1995, respectively. Since 1993, ~10% of the serologic tests for LD have been WBs (table 1). Three laboratories performed almost 60% of the tests for LD on Maryland residents.

Discussion

The use of serologic tests in managing patients suspected of having LD or tick-bite exposures in Maryland is increasing in both number and cost. Our current estimate is that ~30,000 tests, at a cost in excess of $2 million, are performed each year. The bulk of this is for EIA screening tests, which cost about $72 each. However, use of the WB, which costs $106, has increased in recent years and WB tests now constitute about 1 of 10 of the LD serologic tests performed on Maryland residents.

The increase in number of tests ordered and proportion of positive EIA test results over the course of our investigation may be due to changes in LD epidemiology in the state, sensitivity and specificity of LD serology, or LD case management in Maryland.

The number of patients with LD in Maryland reported to CDC has increased each year: from 185 in 1992, to 207 in 1993, to 343 in 1994, and to 336 in 1995. We believe this trend represents an actual increase in patients as well as an increase in reporting [7]. However, it is important to note that CDC-reported Maryland patients with LD account for only 1.1% of the serologic tests for LD performed in the state each year. Therefore, doubling the number of reported patients with LD, as occurred in Maryland between 1992–1993 and 1994–1995, would not necessarily double the number of diagnostic tests.

Following a national conference on LD testing in 1994, the recommendation was made that a two-test approach be used for the serodiagnosis of LD [4–6]. The second National Conference on Serological Diagnosis of Lyme Disease recommendations included lowering the cutoff for a positive EIA test, which resulted in increasing the test’s sensitivity for diagnosing LD at the expense of its specificity. It was also recommended that the laboratory perform a WB on serum samples with positive or equivocal EIA results. Since the WB, as defined in that conference [4–6], was highly specific, this approach should “weed out” the large number of false-positive EIAs resulting from the change in definition of a positive test result. However, many laboratories in Maryland performing the EIA for LD did not do WBs, and there are potential legal consequences if laboratories perform (and charge for) tests that were not ordered by the physician. Therefore, Maryland primary care physicians often retained the responsibility for ordering the second test when the EIA was positive or equivocal, and case management decisions were often made on the basis of results of only an EIA test, often called “a Lyme titer.”

The resulting reduction in cutoff for a positive EIA test could explain the increase in percentage of positive EIA tests between 1992 and 1994–1995. The increase in number and reduced

### Table 1. The use of Lyme disease serology in Maryland: number and type of tests, percentage positive, and cost from 1992 to 1995.

<table>
<thead>
<tr>
<th>Year</th>
<th>EIA No.</th>
<th>Cost ($)</th>
<th>% positive</th>
<th>Western blot No.</th>
<th>Cost ($)</th>
<th>% positive</th>
<th>Total No.</th>
<th>Cost ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992</td>
<td>18,068</td>
<td>1,103,341</td>
<td>3.4</td>
<td>76</td>
<td>6,992</td>
<td>7.9</td>
<td>18,144</td>
<td>1,110,333</td>
</tr>
<tr>
<td>1993</td>
<td>21,901</td>
<td>1,485,656</td>
<td>5.6</td>
<td>2071</td>
<td>220,955</td>
<td>4.4</td>
<td>23,972</td>
<td>1,706,611</td>
</tr>
<tr>
<td>1994</td>
<td>27,701</td>
<td>1,930,708</td>
<td>7.0</td>
<td>3241</td>
<td>343,547</td>
<td>5.0</td>
<td>30,942</td>
<td>2,274,255</td>
</tr>
<tr>
<td>1995</td>
<td>25,753</td>
<td>1,733,269</td>
<td>6.9</td>
<td>2610</td>
<td>277,894</td>
<td>5.5</td>
<td>28,363</td>
<td>2,011,163</td>
</tr>
</tbody>
</table>

NOTE. Total annual cost is based on actual charge for each individual test; 10,168 EIAs performed by Maryland Department of Health and Mental Hygiene for free were priced at $25 each for calculating total cost.
proportion of positive WB tests could also be explained by the reduction in EIA cutoff. The increased number of positive first tests would trigger more second tests being done, which, in turn, would lead to a lower percentage of positive WB tests, since more would be performed on sera from patients whose EIAs were false-positive or equivocal. Also, more stringent criteria for a positive WB following its standardization would result in a lower proportion of these second tests being positive [4–6].

Data from this study and other sources show that physicians in Maryland are using LD serology more often in patient management. They often used EIAs to follow patients after treatment, an inappropriate practice that provides misleading information, increases the proportion of positive EIAs, and increases the overall cost of testing for LD. Also, some physicians have used EIAs and WBs simultaneously while screening for LD. This increases the cost and reduces the proportion of positive WBs. Neither of these practices is considered a cost-effective use of serology in managing LD [4–6].

Due to the large number of EIAs ordered by practicing physicians and the low incidence of LD in the state, the predictive value of a positive first test for LD is low. Let us assume that all cases of LD in the state have EIA diagnostic tests performed to demonstrate this point. If the sensitivity of the test was 92% and its specificity was 82% [5] and there were 300 cases of LD each year (estimate on the basis of official reported cases of LD in Maryland) or there were 3000 cases/year of LD (based on data showing that only 10% of clinical cases of LD in the state are officially reported to CDC [7]), the positive predictive value (PPV) of the EIA would be 0.05 or 0.36, respectively. In both situations, the PPV can be markedly improved by performing a second test on serum from persons with positive or equivocal test results, since the WB has a 98% or 99% specificity [5]. The drawback of this approach is the increased cost. Therefore, it might be cost-effective to base clinical management decisions on results of EIA for patients who have characteristic clinical findings of LD (e.g., facial palsy, arthritis of the knee in children), using the second test only in patients with positive or equivocal EIA results and less typical manifestations of LD.

Previous estimates for the number of serologic tests for LD among Marylanders in 1992 and 1993, calculated from data provided by a survey of a random sample of 1200 physicians, were 30,525 and 36,951, respectively [7]. If our laboratory sample was 80% complete, the corrected numbers of tests performed for those 2 years would be 22,585 and 27,376, respectively, suggesting that the physicians we surveyed overestimated the number of tests they ordered by ~25% each year.

Only one other study has examined the economic impact of LD serology. Ley et al. [8] investigated this in a prepaid health plan in California. Conservatively, assuming a $40 cost for each IFA test performed, they estimated LD serology expenditure to be $16,800 in 1992 and 1993. Furthermore, they found that the majority of tests were not being ordered due to physician suspicion of LD: 35% were requested by the patient, and an additional 41% were ordered as part of screening tests for patients with nonspecific findings. Our results suggest the same practice exists in Maryland, and we join the California investigators [8] in urging primary care physicians to resist using LD serology in this inefficient manner.

Acknowledgments

We thank the commercial laboratories responding to our questions and Alan Fix, Robert McCarter, and Mehret Woubeshet for editorial and technical assistance. Jack K. Grigor was the public health veterinarian responsible for tickborne surveillance in Maryland, and Ebenezer Israel was Director of the DHMH’s Epidemiology and Disease Control Program at the time of this investigation.

References

Invasive Phenotype of Vibrio parahaemolyticus

Yukihiro Akeda, Kenichi Nagayama, Koichiro Yamamoto, and Takeshi Honda

Many studies have been done on the virulence factors of Vibrio parahaemolyticus, which causes acute gastroenteritis. Invasion by this bacterium of culture cells in vitro, however, has not been clearly demonstrated. To assess the invasive ability of V. parahaemolyticus, quantitative studies using antibiotic survival assays were done. Of 21 isolates examined, 4 could invade Caco-2 cells, a human colon carcinoma–derived cell line. Invasion of an isolate, AQ4023, was inhibited by cytochalasin D, nocodazole, and genistein. This indicates that active processes in cells, such as signal transduction by tyrosine protein kinase, may be involved in the internalization of this bacterium by Caco-2 cells and that actin filaments and cytoskeletal structure may have important roles in this process. These results suggested that the disease caused by some isolates of V. parahaemolyticus is attributable not only to toxin production but also to invasion into intestinal epithelium.

Vibrio parahaemolyticus, a causative agent of gastroenteritis, was discovered in 1950 by Fujino et al. [1]. The pathogenesis of this organism has been extensively studied with regard to toxins, especially thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) [2], and adherence factors, including the outer membrane, pili, lateral flagella, and cell-associated hemagglutinin (HA) [3, 4]. However, the overall mechanism of enteropathogenesis of V. parahaemolyticus has not been elucidated.

In the early investigations of V. parahaemolyticus pathogenicity, tissue invasions in vivo were reported by Boutin et al. [5] and Chattarjee et al. [6]: They showed that the organism penetrated into the lamina propria and mucosa of rabbit ileum. Recently, Hlady and Klontz [7] reported that 8% of V. parahaemolyticus infections resulted in primary septicemia and 27% in wound infections. The fact that V. parahaemolyticus can cause septicemia suggests that it can invade the epithelium. However, the invasive capacity of V. parahaemolyticus in vitro has not been studied. Thus, to determine if V. parahaemolyticus can invade Caco-2 cells, 21 isolates (18 clinical and 3 environmental) of V. parahaemolyticus were examined by use of antibiotic survival assays.

Materials and Methods

Bacterial isolates and growth medium. Most V. parahaemolyticus isolates examined in this study were isolated from stools of patients with traveler’s diarrhea at Osaka International Airport Quarantine Station. Three isolates (NAHA-1, THI-4, and THI-6) were environmental isolates. Of 18 clinical isolates 17 possessed the gene encoding TDH or TRH, as determined by polymerase chain reaction [8]; clinical isolate AQ4723 and the 3 environmental isolates did not have the genes. The profile of toxin genes possessed by the isolates is shown in table 1. All clinical isolates but no environmental isolates were positive for cell-associated HA, as determined by a previously described method [4]. The isolates were routinely grown in Marine broth (Difco, Detroit) at 37°C for 18 h. For the invasion assay, isolates were grown at 37°C for 3 h in LB broth containing 3% NaCl, with agitation to midlogarithmic phase. Salmonella typhimurium AQ57498 was used as an invasive control isolate.

Cell culture. Caco-2, a human colon carcinoma cell line, was grown at 37°C in 5% CO₂ in Dulbecco’s modified Eagle minimum essential medium (DMEM) supplemented with 10% fetal calf serum.

Invasion assay. The invasion assay was done essentially as previously described [9]. In brief, Caco-2 cells were seeded onto a 48-well tissue culture plate (10⁶ cells/well), and then 1 μL of bacterial culture diluted to 10⁷ cells/mL was added, and the cells were incubated for 3 h at 37°C in 5% CO₂. The cells were washed twice with sterile PBS (pH 7.0) and incubated for 1 h in DMEM containing 100 μg/mL kanamycin (to kill extracellular bacteria; all the bacterial isolates were sensitive to 100 μg/mL kanamycin). The monolayers were washed once and lysed with 0.1% Triton X-100 in PBS, and cell suspensions were plated onto LB agar containing 3% NaCl to determine the number of intracellular bacteria. When specific inhibitors were incorporated into the assay, they were added to DMEM and were present throughout the assay. Assays were repeated three times, each time in duplicate. Results are presented as the percentages of bacteria internalized, as calculated from the mean number of colony-forming units (cfu) of the bacterial isolate internalized in the Caco-2 cells per the number of bacteria in the original inoculum [9].

Inhibitors. Stock solutions of inhibitors were prepared at the following concentrations in dimethyl sulfoxide: 1 mg/mL cytochalasin D (Sigma, St. Louis), 10 mg/mL nocardazole (Sigma), and 100 mM genistein (Wako Pure Chemical Industries, Osaka). The inhibitors were diluted in DMEM before being added to the Caco-2 cell cultures.

Transmission electron microscopy. Caco-2 cells were grown in a 48-well tissue culture plate (10⁶ cells/well). Bacterial cultures
Table 1. Characterization and invasion potential of \textit{V. parahaemolyticus} isolates against Caco-2 cells.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Toxin (tdh/trh)*</th>
<th>% invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{V. parahaemolyticus} Clinical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AQ4023</td>
<td>trh</td>
<td>9.06 ± 4.50</td>
</tr>
<tr>
<td>AQ4077</td>
<td>tdh</td>
<td>2.97 ± 1.38</td>
</tr>
<tr>
<td>AQ4703</td>
<td>trh</td>
<td>1.86 ± 1.49</td>
</tr>
<tr>
<td>AQ4733</td>
<td>trh</td>
<td>1.81 ± 0.51</td>
</tr>
<tr>
<td>AQ3996</td>
<td>trh</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>AQ4673</td>
<td>trh</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>AQ4674</td>
<td>tdh</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>AQ4680</td>
<td>tdh</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>AQ4686</td>
<td>trh</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>AQ4690</td>
<td>tdh</td>
<td>0.20 ± 0.10</td>
</tr>
<tr>
<td>AQ4696</td>
<td>trh</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>AQ4700</td>
<td>tdh</td>
<td>0.23 ± 0.17</td>
</tr>
<tr>
<td>AQ4713</td>
<td>trh</td>
<td>0.01 ± 0.05</td>
</tr>
<tr>
<td>AQ4723</td>
<td>—</td>
<td>0.43 ± 0.07</td>
</tr>
<tr>
<td>AQ4728</td>
<td>tdh</td>
<td>0.43 ± 0.20</td>
</tr>
<tr>
<td>AQ4750</td>
<td>tdh</td>
<td>0.001 ± 0.001</td>
</tr>
<tr>
<td>AQ4758</td>
<td>trh</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>AQ4761</td>
<td>trh</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>\textit{V. parahaemolyticus} Environmental</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAHA-1</td>
<td>—</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>THI-4</td>
<td>—</td>
<td>0.23 ± 0.12</td>
</tr>
<tr>
<td>THI-6</td>
<td>—</td>
<td>0.01 ± 0.10</td>
</tr>
<tr>
<td>\textit{Salmonella typhimurium}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AQ87498</td>
<td></td>
<td>12.00 ± 3.00</td>
</tr>
</tbody>
</table>

NOTE: *tdh*, gene encoding thermostable direct hemolysin (TDH); *trh*, gene encoding TDH-related hemolysin. Results are no. (mean ± SD) of bacteria surviving incubation with kanamycin, as proportion of inoculated bacteria.

* *Toxin gene profile detected by polymerase chain reaction.

Results

Twenty-one isolates of \textit{V. parahaemolyticus} were examined by use of a kanamycin survival assay (invasion assay) with Caco-2 cells. The results are expressed by percent invasion, as described in Materials and Methods. As determined by the assay, 4 of 18 clinical isolates of \textit{V. parahaemolyticus} had an invasive phenotype (table 1). An isolate was considered invasive if >1% of the original inoculum was internalized. Recoveries of isolates AQ4023, AQ4077, AQ4703, and AQ4733 were 1.81%–9.06% of the original inoculum after infection of Caco-2 cells; isolate AQ4023 showed the highest rate (9.06%). These rates, however, were lower than that for an invasive control isolate (\textit{S. typhimurium} AQB7498; 12%). To confirm the invasion potential of \textit{V. parahaemolyticus}, transmission electron microscopy was used to examine isolate AQ4023: internalized bacterial cells of the isolate were surrounded by the membrane in Caco-2 cells (data not shown).

It has been reported that cytoskeletal and tyrosine protein kinase inhibitors block cell invasion by various pathogenic bacteria [10]. In this study, Caco-2 cells were pretreated with cytochalasin D (actin accumulation inhibitor; 0–1 \textmu g/mL; 30 min), nocodazole (microtubule inhibitor; 0–20 \textmu g/mL; 30 min), or genistein (tyrosine protein kinase inhibitor; 0–200 \textmu M; 10 min) before challenge with isolate AQ4023. As shown in figure 1, internalization of the invading isolate was inhibited...
as a dose-dependent function of inhibitor concentration. In the assay using genistein, the shorter pretreatment time was enough to inhibit invasion. These three inhibitors had no effect on the viability of isolate AQ4023 and its adherence to Caco-2 cells (data not shown).

Discussion

For the study of *V. parahaemolyticus*, most efforts have focused on their toxins, TDH and TRH [2]. This has resulted in only partial clarification of *V. parahaemolyticus* pathogenesis; the exact mechanisms involved in the pathogenesis of this bacterium remain uncharacterized.

We reported herein that some *V. parahaemolyticus* isolates can invade Caco-2 cells. The results of the invasion assays showed that 4 of 21 isolates were highly invasive to Caco-2 cells; 1 was a TDH-producing isolate and 3 were TRH-producing isolates. *V. parahaemolyticus* has generally been considered to be noninvasive, like enteropathogenic *Escherichia coli* [11]. The present finding that some *V. parahaemolyticus* isolates have invasive capabilities is in agreement with the septicemia that occurs in some patients [7]. All clinical isolates examined in this study have cell-associated HA, which was identified as an adhesive factor of *V. parahaemolyticus*; therefore, cell-associated HA-mediated adherence is not always considered to be related to *V. parahaemolyticus* invasion.

A characteristic common to several invasive bacterial pathogens (e.g., *Shigella*, *Salmonella*, and *Yersinia* species and enteroinvasive *E. coli*) is the ability to induce a transient rearrangement of the cytoskeleton of cultured cells during bacterial invasion [8, 12, 13]. To better understand the invasion processes of *V. parahaemolyticus*, we further investigated the invasive mechanism, using agents that inhibit cytoskeletal or signal transduction regulated by tyrosine protein kinases [10, 13]. The results of inhibition assays indicated that all three inhibitors (cytochalasin D, nocodazole, and genistein) reduced bacterial invasion in a dose-dependent manner. These results suggest a plausible role for microfilaments and cellular surface topology in the invasion process: They might provide access to putative receptor sites, such as an invasin-like ligand, or mediate a conformational change in the cytoskeletal structure of the epithelial cell, which would then facilitate endocytosis [14, 15].

To our knowledge, this is the first study to show that *V. parahaemolyticus* has an invasive phenotype in vitro, and this phenotype may relate to *V. parahaemolyticus* pathogenesis in disease. Further detailed study is needed for this determination.

References

Reciprocal Changes in Circulating Interleukin-6 and Its Soluble Receptor during Evolving Sepsis in Leukocytopenic Patients

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Plasma concentrations of interleukin-6 (IL-6) and its soluble receptor (sIL-6R) were serially determined in 32 patients with acute myeloid leukemia who developed severe sepsis (n = 19) or septic shock (n = 13) during chemotherapy-induced leukocytopenia (<1 x 10^9/L). Starting within 2 h of fever onset, IL-6 levels rose significantly over baseline in both groups to markedly higher levels in patients with evolving septic shock (medians: 372 vs. 3671 pg/mL; P < .001). Simultaneously, sIL-6R significantly decreased to lower levels in shock patients than in septic patients without hypotension (53 vs. 93 ng/mL; P = .02). This pattern was maintained throughout the observation period of up to 6 days. In patients with fatal sepsis, peak IL-6 levels were significantly higher than in survivors (P < .001), whereas minimum sIL-6R levels were markedly lower (P = .003). The reciprocal changes in circulating IL-6 and sIL-6R suggest a role for sIL-6R in modulating the effects of IL-6 during evolving sepsis in leukocytopenic patients.

Patients with acute myeloid leukemia (AML) are at high risk of developing severe life-threatening infections during chemotherapy-induced leukocytopenia. Despite the substantial reduction in circulating monocytes and granulocytes, they are capable of mounting a cytokine response during severe sepsis that is similar to that in nonleukocytopenic patients [12]. We have therefore chosen this homogeneous patient population to study the time course and interdependence of circulating levels of IL-6 and sIL-6R during the evolution of severe septic events in leukocytopenia.

Patients and Methods

Patients. Consecutive adult patients with AML who underwent myelosuppressive chemotherapy for remission induction or consolidation were recruited. Patients were excluded if they had evidence of infection before completion of chemotherapy or serious comorbid conditions or did not provide written informed consent to participate. During chemotherapy-induced leukocytopenia (<1 x 10^9/L), patients were monitored for severe septic events as defined by standard clinical criteria [13]. Severe sepsis was diagnosed when fever (>38°C) associated with tachycardia (>90 beats/min) and tachypnea (>20 breaths/min) or hyperventilation (PaCO2 <32 mm Hg) occurred in the presence of suspected or documented infection and if one or more of the following signs of inadequate organ perfusion were present: elevated plasma lactate levels (>1.8 mmol/L), hypoxemia (PaO2/FiO2 ratio <280), oliguria (urine output <30 mL/h or 0.5 mL/kg of body weight/h without corrective therapy), or an acute alteration in mental status. The diagnosis of septic shock was made if, in addition, there was a sustained decrease in systolic blood pressure to <90 mm Hg or of >40 mm Hg from baseline or the need for vasopressor therapy for at least 1 h despite adequate fluid resuscitation and in the absence of other causes for hypotension.

Blood sampling. Baseline plasma samples were obtained prior to the institution of chemotherapy (sample A) and following its completion when the white blood cell count and the absolute neu-
trophil count had decreased to $\leq 1 \times 10^9/L$ and $\leq 0.5 \times 10^9/L$, respectively (sample B). Serial plasma sampling was started within 2 h of the onset of fever. In 19 patients who developed severe sepsis and in 13 patients with septic shock, complete sets of plasma samples obtained at 6, 12, 18, 24, 36, 48, 72, and 144 h after onset of fever were available for the determination of IL-6 and sIL-6R concentrations. For plasma preparation, venous blood was collected into endotoxin-free tubes containing EDTA. Within 60 min of blood sampling, plasma was separated by centrifugation (2000 g, 30 min), aliquoted, and frozen in liquid nitrogen. Plasma samples were stored at $-80^\circ C$ until assayed.

Controls. As controls, plasma samples were collected from 22 healthy subjects (10 men, 12 women) with a median age of 42 years (range, 30–59). IL-6 and sIL-6R determinations. The concentrations of IL-6 and sIL-6R in plasma samples were determined by commercially available EIA as according to the manufacturers’ instructions. The IL-6 assay was obtained from Medgenix (Ratingen, Germany), and the sIL-6R ELISA was purchased from Laboserv (Giessen, Germany). Lower limits of detection were 3 pg/mL for IL-6 and 0.5 ng/mL for sIL-6R. The addition of IL-6 to plasma did not influence the detection of sIL-6R and vice versa.

Statistical analyses. The influence of disease severity (severe sepsis or septic shock) and time on the course of cytokine concentrations was examined by analysis of variance (ANOVA) using log-transformed data. For comparisons of data sets within or between patient groups, the univariate Mann-Whitney U test was employed. The level of significance was set at 0.05, with adjustments made according to Bonferroni when appropriate. Spearman’s correlation coefficient was used to calculate the relationship between IL-6 and sIL-6R levels. Data are presented as medians and interquartile ranges unless otherwise stated.

Results

Thirty-two patients with AML and severe septic events during chemotherapy-induced leukocytopenia were enrolled. Nineteen patients had severe sepsis without hypotension and 13 patients suffered a septic shock, which was fatal in 8 cases. Thus, the mortality rates for all severe septic events and for septic shock were 25% and 62%, respectively. The patient characteristics are shown in table 1.

Median IL-6 plasma levels in AML patients before chemotherapy were slightly higher than in healthy control subjects (27 vs. 5 pg/mL, $P < .001$). However, baseline IL-6 concentrations were not significantly different between patients who subsequently developed severe sepsis or septic shock (medians: sample A, 25 vs. 28 pg/mL, $P = .7$; sample B, 93 vs. 28 pg/mL, $P = .5$). At the onset of fever, IL-6 plasma levels increased in both groups of patients, with significantly higher levels being observed in those with evolving septic shock (medians: 372 vs. 3671 pg/mL, $P < .001$). Throughout the observation period, IL-6 levels in septic shock patients remained distinctly higher than in septic patients without hypotension (ANOVA, $P < .001$; figure 1). Peak IL-6 plasma concentrations in patients with fatal sepsis were significantly higher than in survivors of septic events (figure 1, insert).

Table 1. Patient characteristics according to the severity of septic events.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Severe sepsis (n = 19)</th>
<th>Septic shock (n = 13)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, median (range)</td>
<td>52 (27–77)</td>
<td>55 (35–65)</td>
<td>ns*</td>
</tr>
<tr>
<td>Sex, no. (%)</td>
<td>15 (79)</td>
<td>8 (62)</td>
<td>ns</td>
</tr>
<tr>
<td>Female</td>
<td>4 (21)</td>
<td>5 (38)</td>
<td></td>
</tr>
<tr>
<td>Duration of leukocytopenia before onset of sepsis, days, median (range)</td>
<td>11 (1–37)</td>
<td>27 (4–109)</td>
<td>.025*</td>
</tr>
<tr>
<td>WBCC at onset of sepsis, $\times 10^9$/L, median (range)</td>
<td>0.1 (0.1–0.9)</td>
<td>0.1 (0.1–0.3)</td>
<td>ns</td>
</tr>
<tr>
<td>Blood culture positive, no. (%)</td>
<td>7 (37)</td>
<td>5 (38)</td>
<td></td>
</tr>
<tr>
<td>Gram-positive bacteria</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Microbiologically documented infection, no. (%), total</td>
<td>14 (74)</td>
<td>7 (54)</td>
<td>ns</td>
</tr>
<tr>
<td>Gram-positive bacteria</td>
<td>9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fungi</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. ns, not significant; WBCC, white blood cell count.
* Mann-Whitney U test.
' ' chi-square test.

sIL-6R plasma concentrations in AML patients before chemotherapy did not differ from those measured in healthy subjects (medians: 227 vs. 200 ng/mL, $P = .5$), but a significant decrease was observed in leukocytopenia following completion of chemotherapy (baseline samples A vs. B, $P = .03$; see figure 1). Baseline sIL-6R plasma levels did not differ between patient groups. At the onset of fever, sIL-6R further decreased to significantly lower levels in shock patients than in septic patients without hypotension (medians: 53 vs. 93 ng/mL, $P = .02$). sIL-6R concentrations in shock patients remained lower than those in the severe sepsis group throughout the observation period (ANOVA, $P < .001$). The nadir of sIL-6R levels in patients with fatal sepsis was significantly lower than in survivors (figure 1, insert). Overall, sIL-6R plasma concentrations were inversely correlated to IL-6 levels ($r = -.58, P < .001$).

Discussion

Since the study was conducted in leukocytopenic patients at high risk of developing severe septic complications, we were able to record the levels of circulating IL-6 and sIL-6R from baseline to onset of fever and throughout the evolution of severe sepsis and septic shock. The data demonstrate a rapid rise in IL-6 levels at the onset of septic episodes, with a concomitant decrease in sIL-6R concentrations. This reciprocal pattern was maintained throughout the septic events up to a maximum observation period of 6 days.

Our findings in leukocytopenic patients are in line with the cross-sectional data reported by Frieling et al. [14]. They stud-
pletion of chemotherapy (sample B). This might be explained by the clearance of leukemic blast cells, which indeed have been shown to express IL-6R [15]. However, sIL-6R levels in AML patients before chemotherapy were not different from those measured in healthy subjects, suggesting that the toxic effects of chemotherapy are more likely to account for this observation.

At variance to the report by Frieling et al. [14], our longitudinal data demonstrate that both the increase in IL-6 plasma levels and the decrease in circulating sIL-6R are related to the severity of septic events (severe sepsis vs. septic shock, nonfatal vs. fatal events). Thus, when serially determined, both parameters are markers of disease severity and prognosis in leukocytopenic patients with sepsis. However, whether measurements of either or both parameters will be clinically useful remains to be shown in a prospective setting.

The decrease in circulating sIL-6R coincident with the rise in IL-6 levels may be due to augmented complex formation with the ligand and its subsequent clearance or to decreased generation of sIL-6R in sepsis. The latter could result from diminished expression of sIL-6R or from decreased shedding from the gp80 subunit of the membrane receptor. IL-6R has been shown to be down-regulated after binding to IL-6 and may thus become unavailable for shedding with a consecutive decrease in circulating sIL-6R [16]. As the physiologic regulation of sIL-6R levels is not entirely elucidated, the sepsis-related decrease cannot be explained at this stage. However, it remains important to consider that sIL-6R complexed to its ligand is capable of inducing an IL-6 signal in cells and tissues that do not express the ligand-binding portion of its receptor [8, 10, 11]. Our data therefore support the concept that sIL-6R has a role in modulating the biologic effects of IL-6 in severe sepsis and septic shock.

Figure 1. Time course of plasma levels of IL-6 and its soluble receptor (sIL-6R) is shown for patients with evolving severe sepsis without hypotension (●) and those with evolving septic shock (●). Data are medians and interquartile ranges. A, Baseline determinations before institution of myelosuppressive chemotherapy. B, Baseline measurements in leukocytopenia after completion of chemotherapy. Subsequent samples were collected at onset of fever and at defined intervals thereafter, as indicated. Inserts compare maximum IL-6 and minimum sIL-6R levels measured in survivors (S) and nonsurvivors (NS) of septic events. *P < .001; "P = .003 (Mann-Whitney U test).

References

Association of Recurrent Vaginal Candidiasis and Secretory ABO and Lewis Phenotype

Walter Chaim,* Betsy Foxman, and Jack D. Sobel

The relationship between ABO-Le secretor phenotype and susceptibility to recurrent idiopathic vulvovaginal candidiasis (RVVC) was investigated. ABO and Lewis blood typing was done for 38 women with RVVC (case-patients) and for women in 2 control groups, consisting of 58 healthy women, who were friends identified by case-patients, and 38 race-matched, healthy hospital employees. The 3 groups were similar with regard to age and race. There was no difference in the distribution of ABO phenotype between case-patients and controls. Case-patients were more likely than members of either control group to have Le(a−b0) (nonsecretor) rather than Le(a+b0) (secretor) blood type. With combined nonsecretor Le(a+b−) phenotype and absence of the Lewis gene Le−b+, the relative risk of chronic recurring vulvovaginal candidiasis was 2.41–4.39, depending on the analysis technique and control group. In conclusion, there is an increased frequency of ABO-Le nonsecretor status among women with RVVC.

The etiology of recurrent vulvovaginal candidiasis remains unknown. Only rarely are secondary precipitating or underlying contributory factors identified [1].

The first step in vaginal mucosal colonization by Candida species is adherence to the vaginal epithelial cells [2]. Fucose, which inhibits in vitro binding of yeast to human vaginal epithelial cells [3–5], is also the immunodominant sugar of the H antigen of blood group O and a terminal component of Lewis antigens. H antigen is present in body fluids of secretors of the ABO blood groups, and in 94% of the secretors, there will also be Le(a) and Le(b) antigen. Although vaginal epithelia from women with recurrent candidal vaginitis have not shown increased cell affinity for adherence of Candida species in vitro [6], we hypothesized that secretor status could influence susceptibility to vaginal colonization by Candida fungi. Buford-Mason et al. [7] determined that nonsecretor status was similarly a risk factor for oral carriage of Candida albicans, and Hilton et al. [8] recently reported finding a possible genetic predisposition to recurrent candidal vulvovaginitis. Women prone to infection had a higher frequency of Le(a−b0) nonsecretor phenotype than did healthy controls. The aim of this study was to confirm the relationship between ABO-Le secretor phenotype and susceptibility to recurrent idiopathic vulvovaginal candidiasis.

Materials and Methods

Study participants were selected from patients at the Wayne State Vaginitis Clinic. They were limited to human immunodefi-
cytomegalovirus-seronegative women without diabetes mellitus or immunosuppression, who were not receiving treatment with corticosteroids.

Case definition. Vulvovaginal candidiasis (VVC) was defined as an episode of acute onset of vaginal soreness, irritation, vulvar burning, itching, dyspareunia, vaginal pH between 4 and 4.5, positive saline or 10% KOH wet-mount microscopic examinations, and a positive yeast culture. Women were considered to have recurrent idiopathic vaginitis if they had a documented culture-confirmed episode of acute VVC, history of more than three confirmed episodes of VVC during the year prior to being placed on antifungal maintenance therapy at the clinic, and development of a symptomatic culture-proven episode of VVC while on maintenance antifungal therapy (fluconazole, 100 mg/week; clotrimazole, 500 mg/week) or a relapse of symptomatic and culture-proven VVC within 3 months or two relapses in 6 months after cessation of 6 months of maintenance antifungal therapy. Only patients with infections caused by \textit{C. albicans} were eligible. Patients presenting with mixed infections, including bacterial vaginosis and trichomoniasis, were excluded.

Patient population. Thirty-eight women meeting the case definition were identified (case-patients). Because the clinic is a tertiary clinic, it is difficult to determine the underlying population for study. Therefore, we established 2 control groups: One consisted of friends of the case-patients; the other consisted of hospital employees. It was assumed that friends would be similar with respect to sociodemographic status and behavior, including use of medical services, and would therefore reflect the underlying study population. Each case-patient was asked to identify 3 female friends for participation in the study. Friends meeting study criteria were interviewed by telephone; blood samples were obtained from the women either at the clinic or in their homes. Hospital employees provide a convenience sample of healthy women for comparison and have been used in similar studies. Female hospital employees of the same age and race as case-patients were asked to volunteer; blood samples were collected from those meeting eligibility criteria.

Methods. An antecubital vein blood sample was obtained for ABO and Lewis blood group phenotyping from each study participant. A clotted specimen of whole blood (red tube) was properly labeled and centrifuged for 2 min at high speed. The serum was removed and placed in a second labeled tube. ABO phenotyping (forward and reverse typing) was done by macroscopic hemagglutination: one drop of anti-A, anti-B, and anti-A, B, (Immucor, Norcross, GA) was added to a 4% test cell suspension, which was then centrifuged for 20 s at high speed.

Le(a) and Le(b) phenotyping were done by use of anti-Le(a) and anti-Le(b) murine monoclonal antibodies (Immucor). One drop of 5% cell suspension of test cells was added to a test tube filled with saline for washing of the blood cells, and the tube was centrifuged at high speed for 20 s. The saline was decanted, and one drop of anti-Le(a) added to the test tube. After 5 min of incubation at room temperature and further centrifugation at high speed for 25 seconds, macroscopic agglutination was determined. A similar procedure was done in another test tube with a suspension of 5% cell suspension and a drop of anti-Le(b). The positive and negative controls for phenotyping consisted of cells positive for or lacking the antigen (Gamma Biologicals, Houston), respectively.

### Table 1. Distribution of blood types among women with chronic recurring vulvovaginal candidiasis (case-patients), friend controls, and race-matched hospital employees.

<table>
<thead>
<tr>
<th>Blood phenotype</th>
<th>Case-patients</th>
<th>Friends</th>
<th>Hospital employees</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12 (32)</td>
<td>24 (41)</td>
<td>14 (37)</td>
</tr>
<tr>
<td>B</td>
<td>6 (16)</td>
<td>9 (16)</td>
<td>4 (11)</td>
</tr>
<tr>
<td>O</td>
<td>18 (47)</td>
<td>21 (36)</td>
<td>16 (42)</td>
</tr>
<tr>
<td>AB</td>
<td>2 (5)</td>
<td>4 (7)</td>
<td>4 (11)</td>
</tr>
<tr>
<td>Le(a+b−)</td>
<td>13 (34)</td>
<td>8 (14)</td>
<td>6 (16)</td>
</tr>
<tr>
<td>Le(a−b+)</td>
<td>18 (47)</td>
<td>44 (76)</td>
<td>26 (68)</td>
</tr>
<tr>
<td>Le(a−b−)</td>
<td>7 (18)</td>
<td>6 (10)</td>
<td>6 (16)</td>
</tr>
<tr>
<td>Total</td>
<td>38 (100)</td>
<td>58 (100)</td>
<td>38 (100)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%) of subjects. Distribution of Lewis blood group was significantly different between cases and friend controls ($P = .015$).

Data analysis. Data were entered using Microsoft Excel [9]. SAS software (SAS Institute, Cary, NC) was used for data management and analysis [10]. Exact 95% confidence intervals were calculated around crude odds ratios, using Epi Info (version 6.0; CDC, Atlanta) [11]. For the matched-pair analysis, we calculated the Mantel-Haenszel summary odds ratio, test-based confidence intervals, and the Logit estimator with precision-based confidence intervals, using SAS software [10].

Results

Most case-patients (95%), friend controls (98%), and hospital employee controls (95%) were white. Cases were slightly older (mean age, 39 ± 10 years) than either friend controls (mean, 38.7 ± 8.7) or hospital employees (mean, 37.6 ± 8.1), but the differences were not statistically significant.

There was no difference in the distribution of ABO phenotype between case-patients and either control group (table 1). However, the distribution of Lewis phenotype was statistically different between case-patients and friend controls, with case-patients being more likely to have Le(a+b−) (nonsecretor) than Le(a−b+) (secretor) blood group ($\chi^2 = 4.50, P = .015$). The distribution of Lewis phenotype among hospital employees was similar to that for friend controls; however, it was not statistically significantly different from that for case-patients ($P = .12$), probably because there were only 38 hospital employees, compared with 58 friend controls.

Combining nonsecretor phenotype with absence of the Lewis gene, we estimated risk of chronic recurring vulvovaginal candidiasis for nonsecretors compared with secretors. The risk of recurring vulvovaginal candidiasis for nonsecretors was estimated to range from 2.41 to 4.39, depending on the analysis technique and control group (table 2).

Discussion

In our study, women with recurrent VVC were more likely than friend controls or hospital employee controls to have the
Table 2. Risk estimates for recurrent vulvovaginal candidiasis for nonsecretor, Le(a+b−) and Le(a−b−), compared with secretor phenotype, Le(a−b+), by control group and analysis technique.

<table>
<thead>
<tr>
<th>Control group</th>
<th>OR (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Friend controls (matched analysis)</td>
<td></td>
</tr>
<tr>
<td>Mantel-Haenszel summary OR</td>
<td>4.39 (1.60–12.05)</td>
</tr>
<tr>
<td>Logit OR</td>
<td>2.97 (1.23–7.19)</td>
</tr>
<tr>
<td>Friend controls (unmatched analysis)</td>
<td></td>
</tr>
<tr>
<td>Hospital employee controls</td>
<td>3.49 (1.33–9.20)</td>
</tr>
<tr>
<td>White friend and hospital employee controls</td>
<td>3.21 (1.33–7.73)</td>
</tr>
</tbody>
</table>

NOTE. OR = odds ratio.

nonsecretor phenotype, Le(a+b−) or Le(a−b−). In both control groups, secretor phenotype, Le(a−b+), predominated. Our results are similar to those of Hilton et al. [8]; however, they found a significantly higher frequency of Le(a−b−) phenotypes among women with candidal vulvovaginitis than among hospital employee controls. Our results were not significantly higher, but there were no significant differences between the distribution of Lewis phenotype between our case-patient group and that of Hilton et al. (χ², P = .29), suggesting that the differences may be due to chance. Alternatively, the difference in case definitions used by or sampling discrepancies between the studies might be responsible for the variant conclusion.

To reduce ascertainment bias, we used an extremely strict definition of recurrent idiopathic vulvovaginal candidiasis and selected 2 control groups for comparison. Because case-patients were selected from women seen at a tertiary referral clinic, it was also difficult to determine the underlying population from which they came; thus, we used friends of case-patients as controls. When studying behavioral and sociodemographic characteristics, it is possible that friend controls may overmatch, thus diminishing associations between disease and outcome [12]. However, it is unlikely that case-patients would select friends on the basis of secretor phenotype, except as how this relates to racial group. While a matched analysis resulted in a slightly higher estimate of the odds ratio than an unmatched analysis would, the magnitude and direction of the two estimates were very similar, and the confidence intervals overlapped. The number of participants in the second control group, hospital employees, was smaller; while this negatively affected the precision of the estimate, the odds ratio was again somewhat similar in magnitude and direction to that observed with friend controls.

Our data suggest that Lewis blood group phenotyping may be a marker of patients at risk for recurrent vulvovaginal candidiasis. If the relative risk for recurrent vulvovaginal candidiasis among nonsecretors is 3, this implies that 67% of women with recurrent vulvovaginal candidiasis and nonsecretor phenotype can attribute their vulvovaginal candidiasis to their nonsecretor phenotype. Assuming that 32% of the population has a Lewis nonsecretor phenotype, this phenotype may explain as much as 21% of chronic recurring vulvovaginal candidiasis (67% × 32%). Further studies should examine whether mucosal surface differences as well as those in vaginal secretions explain the susceptibility of some women to repeated bouts of vaginal candidiasis in the absence of other known predisposing factors.

References
The Effects of Inadvertent Exposure of Mefloquine Chemoprophylaxis on Pregnancy Outcomes and Infants of US ArmyServicewomen

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Division of Preventive Medicine, Walter Reed Army Institute of Research, Washington, DC; SRA, Inc., Alexandria, Virginia; US Army Medical Department Activity, Preventive Medicine Service, Fort Campbell, Kentucky

During US military operations in Somalia, mefloquine, a drug for malaria chemoprophylaxis, was not approved for use in pregnant women. Some female soldiers inadvertently used mefloquine before becoming aware of their pregnancy. A registry was established to follow the outcomes of these pregnancies. Questionnaires were administered at the time the pregnancy was diagnosed, after termination or delivery, and at 1 year after birth. Seventy-two soldiers were eligible for the registry. There were 17 elective abortions, 12 spontaneous abortions, 1 molar pregnancy, and 23 live births. The outcome for 19 soldiers was unknown. An unexpected high rate of spontaneous abortions was observed. All infants were healthy at birth, with no major congenital malformations. One infant died at 4 months of viral pneumonitis. At 1 year of age, 13 infants were reported to be healthy, with normal cognitive and motor development. This study provides additional postmarketing data that mefloquine does not cause gross congenital malformations.

There are few antimalarial drugs that are effective and can be used safely for chemoprophylaxis in pregnant women [1]. Mefloquine (Lariam, Hoffman-La Roche, Nutley, NJ), the recommended drug for chemoprophylaxis in most malarious areas because of its effectiveness against chloroquine-resistant Plasmodium falciparum [2], was not initially approved for use in pregnant women. Animal studies indicated some teratogenicity in mice and rats at doses of 100 mg/kg/day [3]. This is much higher than the chemoprophylactic dosage for a 70-kg person of 0.5 mg/kg/day. One study in a refugee population indicated that mefloquine could be safely administered in the latter half of pregnancy (>20 weeks of gestation) [4], but there are few data about its effect on the fetus during the first trimester. Although the risk to the human fetus is thought to be low, there is insufficient information available to establish the absolute safety of mefloquine use during pregnancy [5].

While pregnant soldiers are not deployable, their fetuses may be inadvertently exposed to mefloquine if the soldier has not become aware of her condition before or during the deployment. This situation occurred when female soldiers were deployed to Somalia during Operations Restore Hope and Continue Hope, 1992–1994. All soldiers, except those for whom it was contraindicated, used mefloquine (250 mg each week) for malaria chemoprophylaxis. Loading doses of mefloquine were not used. Before the deployment, female soldiers were advised that mefloquine taken during pregnancy posed a potential risk to the fetus, that they should not become pregnant while on mefloquine, and that pregnancy should be delayed for at least 2 months following the last dose of mefloquine. In March 1993, the Division of Preventive Medicine at Walter Reed Army Institute of Research was asked by the US Army Office of the Surgeon General to establish a registry of all female soldiers who had been deployed to Somalia and had inadvertently taken mefloquine during pregnancy or shortly before conception. In this report, we will describe the outcome of these pregnancies and the health of exposed infants.

Methods

Upon laboratory confirmation of the pregnancy in Somalia, mefloquine chemoprophylaxis was discontinued, and the soldiers were transported to nonmalarious areas. The names and social security numbers of these soldiers were forwarded to the registry. Because of the premature termination of the mefloquine regimen, each soldier was counseled about her risk for malaria. They were told that the risk of congenital abnormalities to the fetus from exposure to mefloquine was thought to be low and that there were no known medical reasons for terminating their pregnancy [6]. Each soldier was also informed of the registry. Army physicians outside Somalia were notified of the registry and were directed to similarly counsel all pregnant soldiers who had been inadvertently
exposed to mefloquine and to forward the soldiers’ names and social security numbers to the registry.

Information about the pregnancy, delivery, and infant was obtained from three questionnaires. The first, given when the soldier was first identified as pregnant, gathered demographic data and mefloquine exposure history. A second questionnaire was administered to collect information on the outcome of the pregnancy. If the soldier carried her baby to term, data were also collected on prior pregnancy histories and outcomes, the current pregnancy and delivery history, and the infant. The third questionnaire was completed 1 year after the birth of the infant and documented the health and development of the child. The questionnaires were completed by health care providers after a chart review or a telephone interview with the soldier. Soldiers were considered lost to follow-up if we were unable after six attempts to contact them by telephone or if the soldier had been discharged from the army.

Results

Seventy-two soldiers were identified as having used mefloquine during their pregnancy. Of these, 17 had elective abortions, 12 had spontaneous abortions, 1 had a molar pregnancy, and 23 had live births. The outcome of the pregnancy was not known for 19 of the 72 soldiers. Of these 19 soldiers, 14 had left the service before information about the pregnancy outcome could be obtained and 2 refused to participate. Three were still on active duty, but information could not be obtained.

The molar pregnancy was diagnosed at 11 weeks of gestation in a black 22-year-old soldier. The ages of the 17 soldiers who had elective abortions ranged from 19 to 32 years (median, 23). Most were not married (n = 12, 71%). Two were married and 1 was divorced. The marital status of 2 soldiers was unknown.

The ages of the 12 soldiers who had spontaneous abortions ranged from 18 to 32 years (median, 22.5). The mean estimated gestational age at the time of the spontaneous abortion was 9.3 weeks (range, 6–12). The average number of mefloquine doses that the fetuses were exposed to was 5.7 (range, 2–12). The total doses of mefloquine taken by soldiers who had spontaneous abortions was similar to that among soldiers who had live births or elective abortions (analysis of variance, P = .12; figure 1). The rate of spontaneous abortions (12/36) in this cohort was 33% (95% confidence interval, 19.5%–49.8%).

There were 23 live births. Fourteen soldiers (61%) were single and 9 were married. Their ages ranged from 18 to 27 years (median, 21). Fourteen soldiers (61%) were primigravida. The average number of mefloquine doses that the fetuses were exposed to was 6.8 (range, 1–16). There were 9 female infants (39%). All of the infants were healthy at birth. Their average 1- and 5-min APGAR scores were 8.1 and 9.0, respectively. The infants’ sizes were appropriate for their gestational age. Their weight ranged from 2693 to 4167 g. There were no congenital malformations. Two abnormalities were noted on the neonatal physical examinations. One infant was jaundiced and the other had a pilonidal sinus.

One infant died at 4 months of age of viral pneumonitis. The cause of death was verified by autopsy, as noted on the death certificate. Health information on 13 other infants was obtained at 1 year of age. All were reported to be healthy and had normal cognitive and motor development. Two of the 13 infants were reported to be small for their ages, but both had small parents. Information was unavailable for 9 (39%) of the infants 1 year after their birth. Most of their mothers had left the army.

Discussion

Our data add further information about the use of mefloquine in pregnant women. We observed no congenital malformations in babies who were exposed to mefloquine in the first trimester, but this conclusion is limited by the small sample size. Our data are consistent with the few reports in the literature. Balocco and Bonati [7] did not find any malformations or perinatal pathologic symptoms in 11 babies born from 10 deliveries to mothers who had used mefloquine during the first trimester. In addition, there were no congenital birth defects reported in a group of 99 travelers whose fetuses had been inadvertently exposed to mefloquine in the first trimester [1].

The rate of spontaneous abortions observed in our registry is higher than that reported in other studies. Among the travelers mentioned above, the rate was 7.6% [1]. In a review of a pharmaceutical database that monitored 331 European women who had inadvertently taken mefloquine during the first trimester, the rate was 9% [1]. This was not statistically different from the rate in women who had used other antimalarial drugs or from a quoted 7%–12% background rate among women with clinically ascertained fetal loss.

There are several possible explanations for the higher rate of spontaneous abortions in our study. It may be a result of our close monitoring of all pregnancies that occurred within our cohort. This prospective surveillance system could note...
early pregnancy losses that might not have been reported in a passive data collecting system. The rate may also be inflated by the high number of elective abortions that occurred. Had these pregnancies progressed to normal deliveries, the denominator for the spontaneous abortion rate would have been greater. Misclassification of the outcomes could have occurred also. Information for some soldiers was self-reported through phone interviews. The soldier may have reported a spontaneous abortion rather than an elective abortion because they believed it to be more socially acceptable. We cannot exclude the possibility that the soldiers in Somalia were exposed to other stresses that could have increased the spontaneous abortion rate. Careful postmarketing surveillance of spontaneous abortion rates should be continued to determine if mefloquine exposure is a causal factor.

Currently, the FDA has categorized mefloquine as a pregnancy category C drug. This means that animal studies have shown an adverse effect on the fetus, but there are no adequate studies in humans; the benefits from the use of the drug in pregnant women may be acceptable despite its risk. Because malaria in pregnant women has a higher mortality rate and greater morbidity than in other adults [8], the risks of malaria in pregnancy may far outweigh any harmful effects of chemoprophylaxis. Recently, the Centers for Disease Control and Prevention (CDC) revised its recommendations for the use of mefloquine in pregnant women, stating that mefloquine may be considered for use if travel to high-risk areas with chloroquine-resistant \textit{P. falciparum} is unavoidable [2]. Continued surveillance of pregnancies is needed to assess adverse reproductive outcomes that might occur at a low rate. Women and health care providers are asked to report the use of mefloquine in the first trimester to the Malaria Section, CDC, telephone (770) 488-7760 for continued assessment of pregnancy outcomes.

Acknowledgments

We thank Mike Thomas and the health care workers of the 86th Evacuation Hospital, the 42nd Combat Support Hospital (CSH), and the 46th CSH for their dedication to duty and support of the registry while working under austere conditions in Somalia and the US Army physicians, physician assistants, and nurses for reporting and obtaining follow-up on eligible servicewomen and their children.

References

Cryptosporidiosis-Induced Impairment of Ion Transport and Na⁺-Glucose Absorption in Adult Immunocompromised Mice

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Electrolyte transport was investigated during chronic cryptosporidiosis in adult anti–interferon-γ–treated SCID mice by means of Ussing chamber techniques. In basal conditions, infection of immunocompromised mice with Cryptosporidium parvum resulted in a 30% reduction (P < .05) in the ileal short-circuit (Isc) current related to a 28% reduction (P < .05) in tissue conductance compared with controls. The rises in Isc and transepithelial potential difference induced by glucose (10 mM) were significantly reduced by Cryptosporidium infection (P < .01) compared with controls. In contrast, responses to mucosal glutamine were marginally affected. Electrical parameters of the ileum were not affected by the addition of indomethacin or furosemide, in either control or Cryptosporidium-infected mice. Thus, long-term cryptosporidiosis in immunocompromised animals leads to a reduction in net ion exchanges, decreased paracellular shunting, and impaired Na⁺-glucose cotransport in the ileum, without prostanoid- or enterotoxin-mediated electrogenic Cl⁻ secretion.

Cryptosporidium parvum is a coccidial protozoan that infects the gastrointestinal epithelial cells of humans and other mammals. The infection can involve all of the gastrointestinal segments but is mainly located in the ileum [1]. In immunocompetent persons, C. parvum infection is uncommon and causes self-limited diarrhea. In contrast, in patients with AIDS, cryptosporidiosis is a frequent opportunistic infection causing unremitting and frequently life-threatening diarrhea [1].

The complex pathophysiology of cryptosporidiosis has been studied mainly in immunocompetent models. Human and animal data suggest that C. parvum infection induces a malabsorptive diarrheal syndrome caused through damage to cells of the villus tip associated with morphologic alterations of the intestinal epithelium and impaired glucose-stimulated Na⁺ absorption [2, 3]. Further studies indicated that the cryptosporidiosis diarrhea is in part of secretory origin. Using a colostrum-deprived neonatal piglet model of cryptosporidiosis, Argenzio et al. [4] detected prostaglandin-mediated secretion of Cl⁻. They have also shown that glutamine enhances electrogenic as well as neutral Na⁺ absorption by a mechanism that involves prostaglandin-sensitive, Cl⁻–dependent, apical Na⁺/H⁺ exchanges [5]. An enterotoxic effect of Cryptosporidium mediated by a heat-labile, calcium-dependent, reversible active factor leading to chloride secretion has also been shown [6].

Many of these data have been obtained during self-limited cryptosporidiosis in neonatal models. The purpose of this study was to investigate electrolyte transport in basal conditions, glucose- and glutamine-dependent NaCl transport during experimental chronic cryptosporidiosis. An adult anti–interferon (IFN)-γ–treated SCID mouse model of cryptosporidiosis was chosen because these animals develop chronic cryptosporidiosis with abundant oocyst shedding [7].

Materials and Methods

Animals and housing. SCID mice (CB-17 scid/scid) were obtained from IFFA CREDO (l’Arbresle, France) and housed in filter-topped microisolation cages in an air-filtered cupboard. They were given sterile food and water, and their cages and bedding were exchanged every week for sterilized ones. They were 6 weeks old at the start of the experiment. Their phenotypic purity was confirmed by the absence of serum IgM.

C. parvum was produced in a high-yield outbred suckling mouse model [8]. Fifteen SCID mice were challenged by gastric intubation with 400 μL of mouse colonic perfusate containing 10⁶ cryptosporidia in 0.025 M PBS, pH 7.2. Ten SCID mice inoculated by gastric intubation with 400 μL of colonic perfusate containing no cryptosporidia were used as controls. One day before inoculation, all mice received an intraperitoneal injection of 125 μg of hamster monoclonal anti-murine IFN-γ. Because this antibody has a half-life of ~1 week, the mice received an additional 62.5 μg of anti-murine IFN-γ 1, 2, and 3 weeks after the initial treatment [7]. Parasite load was checked each week until the day before Ussing chamber studies by counting cryptosporidia in 30 high-power microscopic fields (x200) of modified acid-fast–stained fecal smear.
**Ussing chamber studies.** At week 4 after inoculation, infected and control mice were killed by cervical dislocation and the ileum was removed. Two adjacent segments were rinsed free of intestinal contents, opened along the mesenteric border, and mounted in an Ussing chamber (Marty Technologie, Marcilly sur Eure, France). The mucosal and serosal compartments of the chambers were filled with 4 mL of Ringer’s solution (pH 7.4) containing 140 mM Na⁺, 5.2 mM K⁺, 1.2 mM Ca²⁺, 1.2 mM Mg²⁺, 120 mM Cl⁻, 2.4 mM HPO₄²⁻, and 0.4 mM H₂PO₄⁻ and supplemented with 5 mM sodium pyruvate. Oxygenation of the tissue was ensured by a gas lift of O₂/CO₂ (95:5), and the temperature was maintained at 37°C throughout the experiment. The tissue was short-circuited by an automatic voltage clamp (World Precision Instruments, New Haven, CT). The transepithelial potential difference (PD), measured by using calomel electrodes, and the short-circuit current (Isc) were continuously monitored. Tissue conductance (G) was calculated according to Ohm’s law.

After electrical parameters were stabilized for at least 15 min, Isc, PD, and G were recorded twice at a 5-min interval, and the mean was used as the basal value. Thereafter, for each sample, glucose (final concentration: 10 mM) was added to the mucosal compartment of the first chamber and glutamine (final concentration: 10 mM) was added to the mucosal compartment of the second chamber. The maximal deviation of Isc, PD, and G resulting from glucose and glutamine addition was measured to determine the absorption rate of the nutrients [9]. After 15 min, 10 µM indomethacin or 10 µM furosemide (Sigma, St. Louis) was added to the two compartments to check for possible involvement of prostaglandins and the Na⁺/K⁺/2Cl⁻ cotransport system in the changes in electrophysiologic parameters during cryptosporidiosis.

**Statistical analysis.** For each animal, Isc, PD, and G values are the means of two determinations recorded in the two adjacent segments. Results are reported as means ± SEs of three and four experiments performed with controls and infected animals, respectively. Statistical analysis was done with Student’s t test for unpaired data.

**Results**

**Animal model.** Infection in immunocompromised mice became patent during week 2 (mean number of oocysts per field, ≤1), and there was a rapid increase in oocyst shedding during the next two weeks (parasite score from 1 to 3 to 3+ (>20 oocysts per field, week 4]). None of the anti–IFN-γ–treated mice exhibited clinical manifestations of cryptosporidiosis—diarrhea, wasting, or weight loss—during the study period. No oocysts were detected in control mice.

**In vitro electrical studies.** In basal conditions, infection of immunocompromised mice with *Cryptosporidium* organisms resulted in a significant reduction in the ileal Isc (36.6 ± 3.6 µA/cm²) compared with controls (52.3 ± 2.5 µA/cm²; P < .01) and a significant reduction in G (36.7 ± 4.8 mS/cm² vs. 26.4 ± 1.5 mS/cm² for control and infected animals, respectively; P < .05), whereas the transepithelial PD was only marginally affected (−1.88 ± 0.13 mV vs. −1.57 ± 0.19 mV for control and infected animals, respectively).

Following addition of 10 mM glucose or 10 mM glutamine to the mucosal side of the ileum, Isc and PD rose in both *Cryptosporidium*-infected and control mice, but neither glucose nor glutamine affected G (figure 1). The rise in Isc induced by glucose was significantly reduced by *Cryptosporidium* infection (ΔIsc = 20.0 ± 2.3 µA/cm² vs. ΔIsc = 11.6 ± 1.3 µA/cm² for control and infected animals, respectively; P < .01). After glucose addition, the PD deviation was less in *Cryptosporidium*-infected mice compared with control animals (ΔPD = 0.58 ± 0.28 mV vs. ΔPD = 1.00 ± 0.32 mV for control and infected animals, respectively; P < .05). No significant changes were observed in the PD of ileal mucosa following addition of 10 mM glutamine to either control or infected animals (ΔPD = −0.03 ± 0.10 mV vs. ΔPD = −0.08 ± 0.12 mV for control and infected animals, respectively).
cryptosporidiosis-infected mice (−0.66 ± 0.07 mV) than in control mice (−0.42 ± 0.05 mV; P < .05). The small reductions in glutamine-induced ΔIsc and ΔPD observed in Cryptosporidium-infected mice compared with controls did not reach significance (figure 1).

In neither control nor Cryptosporidium-infected mice were the electrical parameters of the ileum significantly affected by the addition of indomethacin or furosemide (table 1).

**Discussion**

Although *C. parvum* is an important cause of diarrhea in immunocompetent and immunocompromised hosts, the pathophysiology of the infection is poorly understood. Here we report that anti–IFN-γ–treated SCID mice develop mild chronic *C. parvum* infection. In the ileum, cryptosporidiosis was associated with a reduced short-circuit current and tissue conductance, and impaired Na⁺-glucose cotransport, without prostanooid-mediated or enterotoxic electrogenic Cl⁻ secretion.

In contrast with immunocompetent mice, adult anti–IFN-γ–treated SCID mice develop chronic infection with patent histopathologic abnormalities 4 weeks after inoculation, even though there are no clinical manifestations [7]. We thus measured epithelial electrophysiological parameters and nutrient transport in ileal mucosa from control and *C. parvum*–infected SCID mice, by use of Ussing chambers. Our results indicate that in basal conditions, cryptosporidiosis leads to a significant reduction in ileal short-circuit current and conductance, suggesting that both net ion exchanges across the epithelium and the paracellular shunt are reduced. This last observation contrasts with results from in vitro studies showing that the electrical resistance of epithelial cell monolayers decreases as a consequence of *C. parvum* infection [10]; however, it concords with results of studies in neonatal piglets indicating that a reduction in ileal conductance, that is, an increase in ileal resistance, occurs during acute cryptosporidiosis [3, 4]. Tissue conductance is assumed to reflect solute permeation across tight junctions and has been shown to be regulated by the cytoskeleton [11]. In enterocytes, *Cryptosporidium* induces a rearrangement of the cell cytoskeleton, resulting in the formation of a dense band of modified host cytoskeleton proteins underlining the trophozoite-containing vacuoles, which may serve to anchor the parasite to the host cell or, alternatively, resist further invasion into the absorptive cell cytoplasm [12]. This alteration of the host cell cytoskeleton could affect the permeability of the junctional complex and therefore account for the decreased paracellular shunting observed during cryptosporidiosis.

Another important feature of our results is the reduction in the glucose-induced increase in Isc in *C. parvum*–infected mice relative to uninfected animals, suggesting that chronic cryptosporidiosis impairs Na⁺-glucose absorption in immunocompromised mice. In contrast, Na⁺-glutamine cotransport was only marginally affected. A reduction in ileal Na⁺-glucose cotransport has been reported shortly after *C. parvum* challenge in unweaned piglets [2–4]. As the Na⁺-glucose cotransport is mainly expressed in the quiescent enterocytes of the upper half of the intestinal villi [13], reduced villus height is likely to result in smaller number of epithelial transporters and, therefore, in impaired Na⁺-glucose absorption. In contrast, Na⁺-glutamine cotransport appears to occur in differentiated enterocytes as well as in proliferating undifferentiated epithelial cells [14]. This difference could explain why the epithelial response to mucosal addition of glutamine was preserved in our study while glucose absorption was impaired. Such a result has also been reported by Argenzio et al. [5] during acute cryptosporidiosis. Therefore, glutamine could be more effective than glucose in promoting oral rehydration during cryptosporidial diarrhea.

Contrasting with results obtained in neonatal piglets, chronic cryptosporidiosis in immunocompromised mice was not associated with diarrhea. In newborn piglets, Argenzio et al. [4] have shown that an inhibition of ileal NaCl absorption, arising from stimulation of prostaglandin synthesis, occurs during acute cryptosporidiosis. This reduction in NaCl absorption probably plays a major role in *C. parvum*–induced diarrhea. With our model, we failed to observe any involvement of prostaglandins in the alteration of the short-circuit current in *C. parvum*–infected IFN-γ–depleted mice. Marked interspecies variability in the production and involvement of eicosanoids in physiologic and pharmacologic control of intestinal ion transport are known to exist [15] and may account for the discrepancies between our results and those from Argenzio et al. [4]. Moreover, the use of an immunocompromised animal model may also contribute to these differences, as immune cells in the lamina propria are

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**Table 1.** Variations in electrical parameters of ileal mucosa from control and Cryptosporidium-infected immunocompromised mice after addition of 10 μM indomethacin or 10 μM furosemide.

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Indomethacin</th>
<th></th>
<th></th>
<th>Furosemide</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔIsc (μA/cm²)</td>
<td>ΔPD (mV)</td>
<td>ΔG (mS/cm²)</td>
<td>ΔIsc (μA/cm²)</td>
<td>ΔPD (mV)</td>
<td>ΔG (mS/cm²)</td>
</tr>
<tr>
<td>Control (n = 6)</td>
<td>−4.7 ± 2.3</td>
<td>0.17 ± 0.6</td>
<td>0.47 ± 0.61</td>
<td>2.7 ± 1.3</td>
<td>0.07 ± 0.03</td>
<td>−2.37 ± 1.12</td>
</tr>
<tr>
<td><em>C. parvum</em>–infected (n = 10)</td>
<td>−1.3 ± 0.7</td>
<td>0.08 ± 0.02</td>
<td>0.50 ± 0.23</td>
<td>−0.2 ± 1.6</td>
<td>0.05 ± 0.02</td>
<td>0.41 ± 0.75</td>
</tr>
</tbody>
</table>

**NOTE.** Data are means ± SEs. Isc, ileal short-circuit current; PD, transepithelial potential difference; G, tissue conductance.
usually considered to be the main source of eicosanoids in the intestinal mucosa [15].

At present, most experiments on the pathophysiology of C. parvum—induced diarrhea have been done in piglets or calves, as the different attempts to create a rodent model have failed. Our results indicate that although cryptosporidiosis leads to an impairment of ion transport and Na⁺-glucose absorption, it does not trigger diarrhea in IFN-γ depleted mice. This failure is unlikely to arise from reduced intestinal manifestation in mice compared to piglets or calves. Indeed, a large number of oocysts was observed in fecal smears of anti–IFN-γ–treated SCID mice at week 4 after infection. Rather, it seems that a difference in the host response to a similar parasite load is responsible for the differences in the pathophysiology of cryptosporidiosis between mice and piglets. As suggested above, part of this difference may lie in the way epithelial and subepithelial cells produce prostaglandins in response to C. parvum infection.

In conclusion, we provide evidence that chronic cryptosporidiosis in immunocompromised adult mice impairs Na⁺-glucose cotransport and alters net electrogenic ion transport across the intestinal epithelium. Although not reproducing all the symptoms occurring during chronic cryptosporidiosis in immunocompromised patients, this model represents a useful tool to investigate the pathophysiology of C. parvum infection.

References
Differences in Cytokine Responses to *Onchocerca volvulus* Extract and Recombinant Ov33 and OvL3-1 Proteins in Exposed Subjects with Various Parasitologic and Clinical States

Norbert Brattig, Carola Nietz, Sophie Hounkpatin, Richard Lucius, Frank Seeber,* Uwe Pichlmeyer, and Thomas Pogonka

Subjects with generalized onchocerciasis (GEN), with the sowdah form, and with exposure but without onchocerciasis (endemic normal/putatively immune; EN/PI) were studied for cytokine responses to *Onchocerca volvulus* extract (OvAg) and recombinant Ov33 and OvL3-1 proteins. Higher levels of cytokines were produced in response to OvAg in sowdah and EN/PI than in GEN subjects. Peripheral blood mononuclear cells did not produce interferon-γ in response to antigens. OvAg induced interleukin (IL)-5, IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), and soluble IL-2 receptor. EN/PI and sowdah persons produced significantly more IL-5 and IL-2 than GEN subjects, and EN/PI subjects had significantly higher GM-CSF levels than GEN persons. The low IL-5 and GM-CSF levels in GEN subjects were increased by addition of exogenous IL-2. Ov33 and OvL3-1 stimulated production of IL-10 and less IL-5 and IL-2. The study groups did not show a strict Th2-like cytokine response.

Onchocerciasis or river blindness, caused by the tissue-invasive parasitic nematode *Onchocerca volvulus*, is a major cause of blindness and dermatitis in Africa. *O. volvulus*-infected persons exhibit a spectrum of clinical manifestations varying between the common hyporeactive generalized (GEN) form and the chronic hyperreactive sowdah form, occurring in a minority of persons [1]. Sowdah patients show higher levels of antibodies, activated T cells, and eosinophils than GEN patients [1–4]. Recent studies have indicated that T cell responses may be involved in the protection of residents in *O. volvulus*-endemic areas who are exposed to the parasite without developing signs of the disease [1, 5]. These EN/PI (endemic normal/putatively immune) subjects exhibit low levels of *O. volvulus* extract (OvAg)—specific IgG1 and prominent T cell cytokine responses, including the production of interleukin (IL)-2 and interferon-γ (IFN-γ), a profile suggesting protective Th1-type responses [6]. However, several recent experimental studies showed that Th2-like immune responses, comprising the production of IL-4, IL-5, IgE, and eosinophil-mediated effector reactivity, were operative in *Onchocerca* larva–immunized rodents [7].

While previous studies on the cytokine expression pattern have been restricted to the common GEN form, we compared the cytokine responses in the polar forms of onchocerciasis in this study. In addition to the effects of OvAg, we assessed the stimulating properties of two single *O. volvulus* proteins, since in the extract, potential antagonizing activities may obscure responses to distinct *O. volvulus* proteins. The immunodominant Ov33 is of diagnostic relevance [8] and has homology to an aspartyl protease inhibitor of *Ascaris suum* with immunosuppressing properties. OvL3-1 is a muscle protein with homology to zyxin, a cytoskeleton-associated protein of chicken fibroblasts. OvL3-1 have been demonstrated to have B and T cell reactive properties [9].

Materials and Methods

Study population. Forty-six persons from an area hyperendemic for onchocerciasis in Benin, comprising GEN and sowdah forms of onchocerciasis and EN/PI persons, were diagnosed as reported [3]. The EN/PI subjects did not show actual clinical or parasitologic signs nor history of onchocerciasis. They had lived in the area for at least 15 years with exposure to *O. volvulus* similar to that of the onchocerciasis patients. All had negative topical and oral Mazzotti tests with diethylcarbamazine citrate.

Lymphocyte stimulation. Peripheral blood mononuclear cells (PBMC), isolated by density gradient centrifugation, were cultured at $2 \times 10^7$/mL in RPMI 1640 supplemented with 10% fetal calf serum (GIBCO BRL, Eggenstein, Germany) with medium or with one of the following: 1 μg/mL phytohemagglutinin (PHA; Burroughs Wellcome, Großburgwedel, Germany); 10 μg/mL mixture

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Parasitologic and Clinical States

Recombinant Ov33 and OvL3-1 Proteins in Exposed Subjects with Various mental studies showed that Th2-like immune responses, protective Th1-type responses [6]. However, several recent experi-
of nonparasite antigens composed of tetanus, tuberculin, streptoly-sin-O, vaccinia, and mumps antigens (Behringwerke, Marburg, Germany) [3]; 1 µg/mL OvAg; recombinant Ov33; OvL3-1; or equivalently expressed dihydrofolate reductase (DHFR) [9a].

Recombinant antigens Ov33 and OvL3-1 were expressed in Escherichia coli using the plasmid pDS56/RBSI, 6xHis, giving rise to polypeptides with a leader sequence of 6 histidines at the N-terminus [10]. Since the full-length Ov33-3 cDNA (European Molecular Biology Laboratory [EMBL] accession no. X13313) could not be expressed, a truncated version, lacking the 14 C-terminal amino acids, was used. The complete OvL3-1 cDNA (EMBL accession no. X60100) was applied to express the recombinant protein. Both recombinant proteins were purified by Ni۪۪+ chelating chromatography. Polypeptides were dialyzed against PBS and sterilized by filtration. As a control, mouse DHFR containing an N-terminal histidine leader sequence was similarly expressed and purified. No endotoxin (< .01 U/mL; E-Toxate test; Sigma, St. Louis) was detected in these recombinant antigen preparations. The recombinant antigens failed to stimulate detectable production of IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), and soluble IL-2 receptor (sIL-2R) in cells from 7 of 8 nonendemic volunteers. In a number of experiments, recombinant human IL-2 (Boehringer Mannheim, Mannheim, Germany) was added to the cell cultures at a final concentration of 500 or 1000 pg/mL in the absence or presence of OvAg. Due to limitations on the numbers of PBMC, not all experiments could be performed for each participant.

Measurement of cytokine secretion. Supernatants of the cultured PBMC were collected after 2 days for quantification of IL-2, IFN-γ, IL-4, IL-10, and sIL-2R and after 5 days for IL-5 and GM-CSF. IL-4, IL-5, IL-10, and GM-CSF were measured by capture ELISA using pairs of capture and detecting monoclonal antibodies according to the manufacturer’s protocol (Pharmingen, Hamburg, Germany). The concentrations of the cytokines were calculated by interpolating standard curves. IFN-γ, IL-2, and sIL-2R were measured by use of commercial ELISAs (Dianova, Hamburg, Germany). IFN-γ values are expressed as units; all other cytokines are measured in picograms per milliliter. Net production of cytokines was calculated by subtracting the values for spontaneous release from those of the stimulated cultures. Cytokine production in response to the recombinant proteins was corrected by the low background values obtained for cultures containing control DHFR, thereby excluding putative stimulatory effects by bacterial endotoxin. Increased spontaneous release was found for IL-10, GM-CSF, and sIL-2R.

Determination of IgG isotypic antibody to OvAg. IgG was analyzed with a modified ELISA procedure as described [3]. The amount of IgG isotype was calculated by use of the titration-curve method in arbitrary units representing the titer of positive reaction (above cutoff level), determined by linear regression. IgG2 isotypic antibodies were not included because this isotype predominantly recognizes cross-reacting phospholipids and carbohydrates.

Statistical analysis. Data of the study groups (table 1) are given as medians and quartiles. The cytokine concentrations are presented as dot plots supplemented with the associated medians (figure 1). Differences in the cytokine concentrations between the 3 study groups were analyzed using the exact Kruskal-Wallis test with Bonferroni-Holm adjustment within the subsets of data obtained by OvAg and the recombinant proteins. To test for significance, the Wilcoxon test for unpaired samples with Bonferroni-Holm correction was applied to compare GEN versus sowdah and EN/PI groups, respectively. The experimental type I error rate was set to 5%.

Results

Clinical and serologic data characterizing the study groups are shown in table 1. GEN and sowdah patients had significantly higher IgG1 and IgG4 antibody titers to OvAg than EN/PI subjects (P < .01). Sowdah patients had significantly higher IgG3 titers to OvAg than EN/PI subjects (P < .05). EN/PI persons, however, showed significantly higher IgG1 titers to OvAg than nonendemic controls (P < .01; data not shown).

After stimulation with OvAg, PBMC from all study groups produced IFN-γ (figure 1A). IL-4 and IL-10 were only sporadically detected. Higher levels of IL-2, sIL-2R, IL-5, and GM-CSF were found in cell culture supernatants from sowdah patients and EN/PI persons than from GEN subjects, showing a similar pattern of cytokine production. Multiple comparison procedures showed that IL-2 and IL-5 were generated at significantly higher levels in sowdah patients (P = .04 and P = .02, respectively) and EN/PI persons (P < .01) than in GEN subjects. Similar to levels of IL-2, the lowest levels of sIL-2R were found in the GEN group. OvAg-induced GM-CSF levels were significantly higher in EN/PI than in GEN subjects (P < .01). The highest cytokine levels were observed in sowdah patients, who had the highest IgG1 and IgG3 levels to OvAg (data not shown).

The low levels of IL-5 and GM-CSF produced in GEN patients could be elevated by supplementing IL-2 into the cell cultures. IL-5 and GM-CSF, but not IL-4 and IL-10, were measured at significantly higher concentrations for all 3 study groups (P = 02). This IL-2-dependent production of IL-5 and GM-CSF was lower in GEN than in EN/PI persons (IL-5: 110 [range: 10–250] vs. 212 [range, 67–450] pg/mL, P = .04; GM-CSF: 125 [range, 30–220] vs. 430 [range, 365–580] pg/mL, P = .02).

Stimulation of lymphocytes with Ov33 and OvL3-1 induced no significant IFN-γ, IL-2, or IL-4 levels. In contrast to OvAg, both proteins induced IL-10 but less IL-5 and no IL-2 (figure 1B, C). In response to Ov33, EN/PI persons generated significantly higher levels of sIL-2R (P < .01) and marginally higher IL-10 and GM-CSF levels than GEN patients. In some experiments, the cells were stimulated with higher concentrations of Ov33 (5 µg/mL), which resulted in markedly higher levels of IL-10 in the culture supernatants (data not shown). IL-5 responses to OvL3-1 were significantly lower in GEN than in sowdah or EN/PI persons (P < .01). In GEN subjects, however, the highest GM-CSF levels were observed with OvL3-1.

After polyclonal stimulation of PBMC with PHA, cytokine levels did not differ significantly in the 3 study groups, with the exception of IFN-γ, which was higher in the EN/PI than the
Table 1. Demographic, parasitologic, and serologic data of the groups in the *O. volvulus* cytokine response study.

<table>
<thead>
<tr>
<th></th>
<th>Generalized</th>
<th>Sowdah</th>
<th>EN/PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>30</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Sex, no. male/female</td>
<td>16/14</td>
<td>5/1</td>
<td>4/16</td>
</tr>
<tr>
<td>Age, years, medians</td>
<td>35 (25–40)</td>
<td>38 (27–42)</td>
<td>23 (17–28)</td>
</tr>
<tr>
<td>Microfilarial density,</td>
<td>13 (4–48)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Intestinal nematodes,</td>
<td>67</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>Anti-<em>O. volvulus</em> titer, U/mL (range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG1</td>
<td>4300* (3800–5500)</td>
<td>5250* (3200–6450)</td>
<td>3050 (2900–3300)</td>
</tr>
<tr>
<td>IgG3</td>
<td>1900 (1050–3000)</td>
<td>3300² (1000–4700)</td>
<td>0 (0–1600)</td>
</tr>
<tr>
<td>IgG4</td>
<td>4500* (3900–5400)</td>
<td>4450* (3350–5550)</td>
<td>700 (0–1000)</td>
</tr>
</tbody>
</table>

NOTE. EN/PI, endemic normal/putatively immune.
* *P* < .01 vs. EN/PI.
² *P* < .05 vs. EN/PI.

**Discussion**

This study demonstrated, first, that the cytokine production in response to OvAg was greater in the onchocerciasis-free *O. volvulus*–exposed EN/PI group and the hyperreactive sowdah group than in the GEN onchocerciasis group. In GEN subjects, only IL-10 and GM-CSF were detected at higher levels, predominantly after stimulation with the recombinant proteins. Second, the demonstration of IL-5 and IL-10 production, in the absence of IFN-γ, may indicate a Th2-like cytokine response [11–13]. Experiments with isolated CD4 cells were not feasible in this investigation, which was done in a field station, precluding a statement about the origin of the cytokines. The failure to detect IL-4 after stimulation with filarial antigens has been observed frequently [6, 11–13] and may indicate a consumption rather than absence of IL-4. Third, an interrelated pattern of cytokine production, recently reported for EN/PI persons in response to *Wuchereria bancrofti* [13], was indicated by the generation of IL-2 and sIL-2R, in addition to IL-5 and IL-10, and by the IL-2 dependence of IL-5 production, as has been reported once before [11]. Finally, whereas IL-5 was prominently produced in response to OvAg, IL-10 was measured at higher levels after stimulation with Ov33 and OvL3-1.

The low level of cytokine production in the GEN group in response to OvAgs is in accordance with earlier studies demonstrating impaired T cell responsiveness to OvAg in GEN subjects [1, 5, 14]. However, cells from GEN persons were responsive, as they produced most cytokines normally after activation by PHA (not shown). Only the Th1 response—promoting IFN-γ production was reduced (data not shown). PBMC of GEN subjects produced IL-2 after stimulation with nonparasite antigens (data not shown) but not to OvAgs. The low IL-5 response to OvAg could be increased by supplementation with IL-2. The microfilaria-positive GEN patients express an inappropriate IL-2 response to OvAgs, which may be responsible for the low IL-5 response. Consistent with these results, Steel and Nutman [11] observed an IL-2 dependency in the expression of IL-5 mRNA. We found that neutralizing antibodies to IL-2 strongly reduced the production of IL-5 (data not shown). IL-2, therefore, seems to be an important activator of the Th2 response in this helminth infection. In response to OvAg, cells of sowdah patients and EN/PI persons prominently produced IL-5 and GM-CSF, both cytokines that regulate eosinophils and basophils, two major Th2-associated effector cells in helminth diseases.

In our study, sowdah patients produced substantial levels of IL-5, GM-CSF, and IL-2/sIL-2R, that is, cytokines that activate effector cell mechanisms. The cytokine response was particularly pronounced in sowdah patients with pathologic symptoms and high antibody levels. These findings, in this subset of the rare sowdah form of onchocerciasis, have to be confirmed. They indicate a possible relationship among Th2 cell activation, effector responses, and low parasite load [7]. Reports of several studies have described the elevation of eosinophils, IgE, and IgG in sowdah patients [1–4]. In these patients, onchocercomata and lymph nodes are enlarged and harbor higher numbers of inflammatory cells, such as eosinophils and macrophages. At the same time, low numbers of *O. volvulus* adults and microfilariae are present in these patients compared with GEN subjects [15]. Thus, strong proinflammatory indicators parallel low parasitic burden. Ottesen [1] discussed that sowdah patients have heightened responsiveness to microfilariae, tolerate them less well, and attack them more aggressively or that the inflammation-limiting mechanisms are relatively deficient in sowdah.
Figure 1. Cytokine responses to *O. volvulus* adult antigen (OvAg; A), recombinant Ov33 (B), and OvL3-1 (C) in patients with generalized onchocerciasis (GEN), sowdah, and endemic normal/putatively immune persons (EN; see Materials and Methods). Cytokine levels (in U/mL for IFN-γ and pg/mL for all other cytokines) are shown as dot plots and medians analyzed for statistical significance by Kruskal-Wallis and Wilcoxon tests with Bonferroni-Holm correction. * Significant differences. A, GEN patients generated, in response to, OvAg significantly lower IL-2 and IL-5 levels than sowdah patients (P < .04 and P < .02, respectively) and EN subjects (P < .01) and showed lower GM-CSF levels than EN subjects (P < .01).

B, Ov33-induced levels of sIL-2R were significantly higher in EN than in GEN patients (P < .01).

C, OvL3-1–induced levels of IL-5 were significantly higher in EN and sowdah subjects than in GEN patients (P < .01).

In contrast to GEN subjects, but similar to the sowdah group, the EN/PI persons demonstrated prominent cytokine responses, predominantly of IL-2/sIL-2R, IL-5, IL-10, and GM-CSF. Exposure of these residents to *O. volvulus* is similar to that of both groups of patients with onchocerciasis, but EN/PI subjects appear free of adult and microfilarial stages. Elson et al. [6] reported a Th1-like cytokine response characterized by IFN-γ and IL-2 in a proportion of EN/PI subjects. However, in the latter study, this subgroup of EN/PI subjects produced higher levels of IL-5 and IL-10 than the rest of the EN/PI subjects, as well as the microfilaria-positive subjects, upon stimulation with OvAg [6]. Our results are in accordance with recent data suggesting that *O. volvulus*–infected and EN/PI persons have no strict Th1/Th2 dichotomy and that EN/PI subjects, showing a relative hyperresponsiveness for IL-2, IL-5, and GM-CSF, exhibit no predominance of Th1-type cytokine responses [11, 13, 16].

The Th2-like immune response elicited by Ov33 was substantiated in our recent study on T cell proliferation and antibody responses toward Ov33 protein and three Ov33 polypeptides. We demonstrated Ov33 and Ov33 peptide-specific IgG4 and IgE antibodies in GEN persons and, in a number of cases, production of IL-4 and IL-10 in response to the Ov33 polypeptides [9a]. In a recent report, specific IgE and IgG4 antibody responses were also shown for OvD5B, an analogue to Ov33 [16].

Our results support the view that the investigated *O. volvulus* antigens elicit a mixed pattern of Th cytokine responses in onchocerciasis. These appear to be more pronounced in sowdah and EN/PI subjects than in GEN patients and may be operative in pathogenic or protective mechanisms.
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