CONCISE COMMUNICATIONS

Tissue Distribution of Hantavirus Antigen in Naturally Infected Humans and Deer Mice

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The Sin Nombre virus (SNV) is the etiologic agent of hantavirus pulmonary syndrome in humans but does not cause disease in chronically infected deer mice *(Peromyscus maniculatus)*, the natural host. In this study, murine antiserum raised against recombinant SNV nucleocapsid protein was utilized to localize viral antigen immunohistochemically in tissues from both humans (n = 20; 11 positive, 9 negative) and deer mice (n = 6; 4 positive, 2 negative). Viral infection status was confirmed by Western blot or reverse transcriptase–polymerase chain reaction. SNV antigen was detected in pulmonary and cardiac endothelium in both species, but positive cells in deer mice were rare. Other deer mouse tissues, including kidney, were negative; in contrast, vascular elements of several tissues from infected humans were positive, with strong staining of renal endothelium. The paucity of positive cells in chronically infected mice suggests a low virus burden compared with that of acutely infected humans.

Hantaviruses, which constitute a genus of the Bunyaviridae family, are primarily harbored by rodents. Hantaviruses do not appear to cause disease in their rodent hosts but are considered the etiologic agents of two distinct disease syndromes in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Of these, HFRS is associated with hantaviruses carried by murid rodents of Old World origin, whereas HPS is caused by hantavirus species carried by sigmodontine rodents indigenous to the Americas [1].

HPS was first identified in the spring of 1993, when a cluster of cases in the Four Corners area of Arizona, Colorado, New Mexico, and Utah brought attention to the disease. As of July 1997, there have been 167 cases of HPS reported, with 47% mortality. Sin Nombre virus (SNV) is endemic in the deer mouse *(Peromyscus maniculatus)* in western North America and is the predominant agent of HPS in North America [2, 3]. Reliable diagnostic assays for SNV antibodies and genetic materials are available. The assays (Western blot, strip immunoblot assay, and reverse transcriptase–polymerase chain reaction [RT-PCR]) require laboratory personnel to handle potentially infectious material (patient serum, blood, or tissue), but they have very high sensitivity and specificity for acute SNV infection [4–6]. An immunohistochemical assay utilizing antibody directed against the SNV nucleocapsid (N) protein of a related hantavirus has been used to detect viral antigen in formalin-fixed tissues from HPS patients for retrospective diagnosis of HPS and to further elucidate the pathogenesis of this disease [7, 8].

The purpose of this study was to demonstrate SNV tissue localization in human patients and in seropositive deer mice, using an antibody directed against an SNV protein. This study differs from previous studies in the immunogen used for antibody development. Ultimately, we hope to contribute to the understanding of the mechanisms of SNV transmission, the pathogenesis of HPS in humans, and the absence of clinical disease in rodent carriers.

Materials and Methods

Patient samples. Formalin-fixed autopsy tissue from 11 patients who died of HPS during the spring and summer of 1993 were obtained through the New Mexico Office of the Medical Investigator and the University of New Mexico Department of Pathology. In all cases, infection with SNV was confirmed by the characteristic history, progression, and pathologic findings associ-

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Informed consent was obtained. Human experimentation guidelines of the US Department of Health and Human Services and the University of New Mexico Institutional Review Board were followed in the conduct of this research.

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ated with the disease, as well as by positive IgM and IgG antibody reactivity to recombinant SNV-N protein and SNV G1 glycoprotein by Western blot assay. The presence of SNV RNA was confirmed by RT-PCR in all cases for which appropriate tissue or blood samples could be obtained (6/11 cases). All 11 cases are recognized as HPS patients by the US Public Health Service. Tissues from an additional 9 patients with nonhantaviral pulmonary disease were chosen as negative controls. These patients died of non-AIDS–related pneumonia ($n = 5$), AIDS-related pneumonia ($n = 2$), or interstitial pulmonary fibrosis ($n = 2$).

Peromyscus maniculatus samples. Serum samples from an established colony of wild-caught *P. maniculatus* were screened by Western blot for antibody reactivity to recombinant SNV-N proteins. Of 80 animals screened, 8 were seropositive. These animals were culled from the colony and sacrificed. Four of the seropositive animals were injected intrapleurally and intraperitoneally with a total of 5 mL of 10% neutral-buffered formalin, and tissues were collected and processed for histochemistry within 48 h. The remaining seropositive animals were frozen for RT-PCR and viral isolation studies. Nucleotide sequence analysis of amplified viral cDNA from 3 specimens confirmed that SNV was the hantavirus within the colony. There were essentially no differences in the N protein amino acid sequences for the different mouse and human samples analyzed (Hjelle B, unpublished results, 1994). The colony was rescreened 1 month later, and all animals were seronegative. Two animals were sacrificed, injected with formalin, and processed as above as negative controls for immunohistochemical studies.

Development of primary antibody. Full-length SNV-N protein cDNA was cloned into an *Escherichia coli* expression vector (pMAL-c2; New England BioLabs, Boston, MA), and the N protein was expressed as a fusion protein with maltose binding protein and then purified over an amylose resin column (New England BioLabs) according to previously described methods [9]. Mature BALB/c mice were immunized intraperitoneally with 50–100 μg of SNV-N/pMAL fusion protein in emulsion containing Freund's complete adjuvant (Sigma, St. Louis). Immunizations were repeated 3 times at 14-day intervals with 50–100 μg protein in Freund's incomplete adjuvant. Sera from immunized mice were periodically tested for specific immunoreactivity to SNV-N by ELISA using SNV-N antigen [5]. Serum was collected from 2 strong immunoreactors, and pooled serum was utilized as the primary antibody throughout this study.

Immunohistochemistry. For all tissues sampled, 4- to 6-μm sections were mounted on glass slides, deparaffinized, and stained on an automated processor following antigen retrieval. Antigen retrieval involved boiling tissue sections for 19 min in 0.5 *M* citrate buffer, pH 6.5, after which slides were cooled in citrate buffer for 20 min immediately prior to staining.

Since a secondary anti-mouse conjugate was used in our studies, we needed to consider possible antigenic cross-reactivity due to species similarity between *Mus musculus* and *Peromyscus* species. A prestaining step to block endogenous *P. maniculatus* immunoglobulin prior to the addition of primary antibody was necessary to avoid high background reactivity for all *P. maniculatus* tissues (Histomouse kit; Zymed, Camarillo, CA, used according to the manufacturer's instructions). Without this blocking step, nonspecific staining in all mouse tissues evaluated (both seropositive and seronegative) precluded meaningful interpretation of results. As a control for possible effects of this blocking agent on specific detection of SNV antigen, a subset of the human tissues was stained with and without the prestaining block: no significant difference was noted (data not shown).

Immune mouse serum was applied at 1:2000, 1:4000, and 1:16,000 dilutions. Next a biotinylated goat-anti mouse secondary antibody was applied, followed by a horseradish peroxidase–avidin conjugate. Positive staining was visualized utilizing an amino–ethyl carbazole chromogen. Stained slides were counterstained with hematoxylin, mounted with aqueous mounting media, and viewed at ×100–1000.

Results

All slides were reviewed by 3 independent readers who were blinded to the clinical status of each patient. Discrepancies in results were rare and were resolved by the readers by use of a multiheaded microscope. For all slides, cell staining was graded as negative, focal, multifocal but sparse, or widespread. Results are summarized in table 1. In seropositive patients, N-antigen localized to the endothelium in a dense, granular-to-punctate, dark red staining pattern (figure 1A–C.) In the human tissues evaluated, the specific staining was readily distinguished from nonspecific staining, which tended to be more orange and globular with indistinct margins, and from endogenous pigments, such as lipofuscin. Pulmonary endothelium demonstrated the strongest staining of the tissues examined, as evidenced by the intensity and abundance of signal within individual cells, and by the widespread distribution of positive cells throughout the tissue. Positive pulmonary endothelial staining was evident in all HPS patients examined for whom lung tissue was available (10/11 patients). Viral antigen in other tissues was most consistently found in the endothelium, but variable staining of hepatic Kupffer cells, splenic dendritic cells, circulating mononuclear cells, and pulmonary macrophages was also observed. Staining of renal glomerular capillary endothelium was prominent as was staining of myocardial endothelium in some patients. The staining patterns that we observed largely parallel those reported using the heterologous antibody [7].

In tissues from infected *P. maniculatus,* viral antigen staining was demonstrated only in pulmonary and cardiac tissues. In general, viral antigen–positive cells had few dark red punctate signals, and individual positive cells in these tissues were rare for all animals examined. Unexpectedly, no viral antigen was detected in the kidney of seropositive animals. We determined that antigen detection was not obscured by the blocking reagent and attribute the higher background staining in the human tissues to species differences or differences in tissue processing (mice were formalin-fixed immediately following sacrifice). Even with the blocking step, there was widespread intravascular orange-pink background staining of serum in both seropositive and seronegative animals. No positive (punctate) staining

Table 1. Results of cell staining of various formalin-fixed autopsy tissues from 11 patients who died of hantavirus pulmonary syndrome (HPS), 9 patients with nonhantaviral pulmonary disease (negative controls), and 6 wild-caught *Peromyscus maniculatus*.

Patient no., sex	Age (years)	Lung pathology	Immunoblot	RT-PCR	Lung	Heart	Kidney	Liver	Spleen	Lymph node	Pancreas	Intestine	Salivary gland	Adrenal	Ovary
HPS patients															
2257, F	31	HPS	+						N	F		F			
2717, F	21	HPS	+		W		W		W						
2718, M	19	HPS	+		W			N	N						
2823, F	58	HPS	+		W		W	F							
2891, M	21	HPS	+	+	W										
3053, F	22	HPS	+	+	W	N		N	W			F			N
3188, F	64	HPS	+	+	W			F	W	N				F	
3226, F	39	HPS	+	+	W	F	W		F						
3326, F	42	HPS	+		W					N			N		
3376, M	34	HPS	+	+	W	W		F			W				
3472, M	68	HPS	+		W	N	W	N	N	N					
Negative controls															
2732, F	56	NARP			N		N								
1530, F	81	NARP			N										
1913, M	35	NARP			N		N								
3029, F	61	NARP			N										
5074, F	74	IPF			N										
2736, M	34	ARP			N										
2332, M	41	ARP			N										
5374, M	42	DAD			N			N			N				
1660, F	51	DAD			N										
P. maniculatus															
H2A			+		F	F+	N	N			N				
H2D			+		F+	F	N	N	N		N				
H4B			+		F	F+	N	N	N		N				
H4E			+		F	F	N	N							
NN1			−		N	N	N	N	N		N				
NN2			−		N	N	N	N	N						

NOTE. RT-PCR = reverse transcriptase–polymerase chain reaction. + = positive, N = negative, F = focal, W = widespread, NARP = non-AIDS–related pneumonia, IPF = interstitial pulmonary fibrosis, DAD = diffuse alveolar disease, F+ = multifocal but sparse, − = negative.

was detected in any of the tissues from negative control animals. These data are summarized in table 1. Characteristic staining of pulmonary and cardiac endothelium, as well as negative renal endothelium from a seropositive animal, are shown in figure 1D–E.

Discussion

Hantavirus-related disease has occurred as early as 1959 in the New World [10]. However, HPS in humans was first recognized during an outbreak in the spring of 1993. Sporadic cases of HPS have occurred in the past and have gone unrecognized. The development of an immunohistochemical assay for retrospective diagnosis of such cases is a useful tool for investigating the natural history of HPS in humans [8, 11], as are carefully designed serologic tools with epitope mapping [12, 13].

In the development of such an assay, one must consider the potential risks involved in working with live virus or infected animals. We used formalin-fixed tissue in this study to eliminate the risk of virus exposure to all personnel who might come into contact with either the tissue blocks or slides before they were completely processed. In addition, the use of recombinant viral protein as an immunogen both defines the targeted antigen and ensures that immunized animals do not pose an infectious threat to laboratory personnel.

In this study, we have shown that the use of murine polyclonal antisera generated against a recombinant full-length SNV-N protein demonstrates promise as a useful indicator of SNV infection in humans, demonstrating similar specificity to that of an assay based upon a monoclonal antibody to a related hantavirus (CDC monoclonal antibody GB04-BF07 directed against Puumala hantavirus nucleoprotein [7]). The human tissues we evaluated had undergone various degrees of autolysis or processing (or both; e.g., embalming) prior to formalin fixation. Nevertheless, antigen was readily detected at antibody dilutions of 1:16,000. None of the tissues examined from seronegative human tissues demonstrated antigen staining.

Although only a few seropositive *P. maniculatus* were examined in this study, the findings were consistent among these samples. The rodent hosts of SNV manifest few if any of the

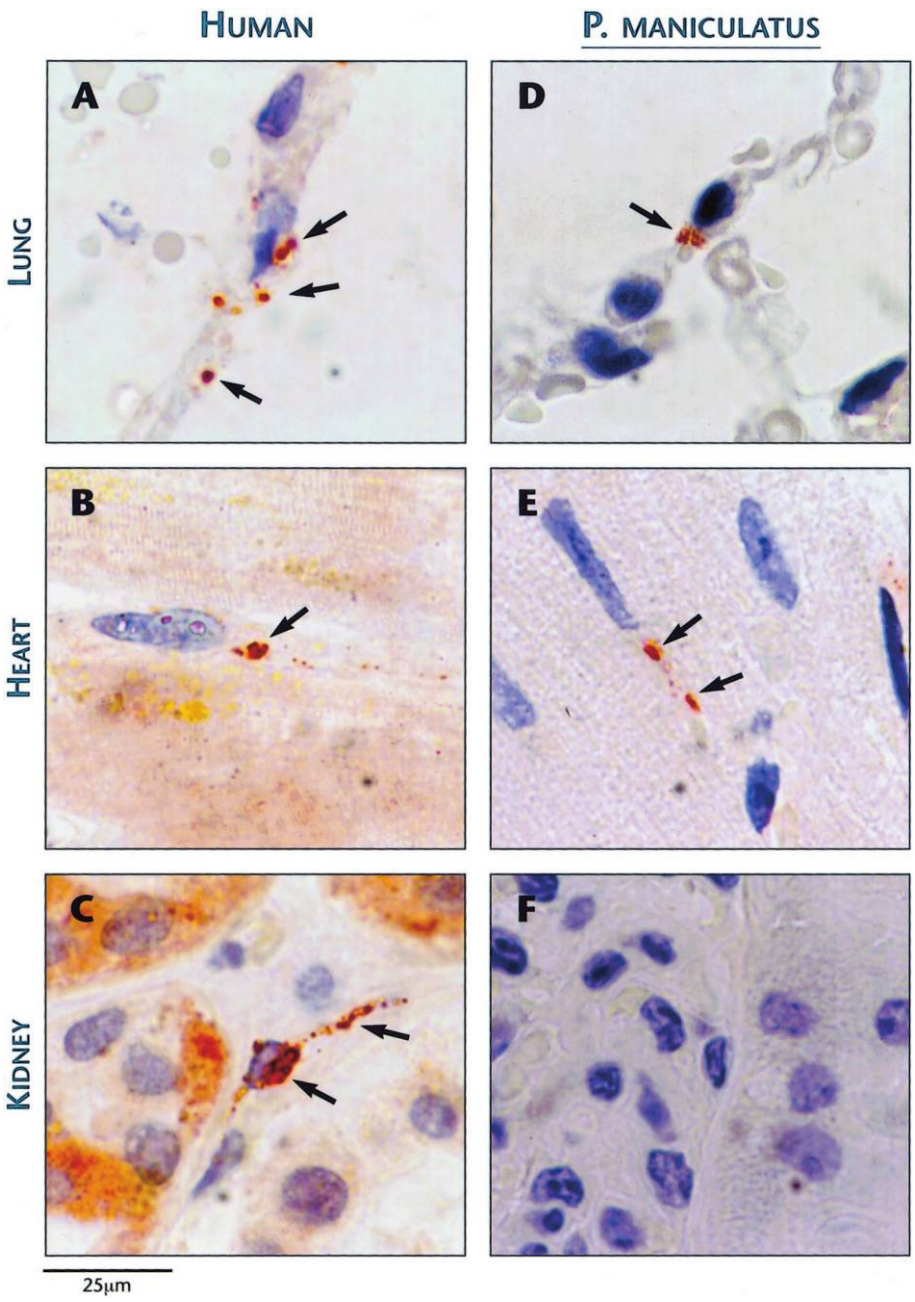

Figure 1. Immunohistochemically stained tissues from Sin Nombre (SNV) virus–positive humans and deer mice. **A**, Human lung: alveoli with antigen-positive capillary endothelium. **B**, Human heart: ventricular myocardium with antigen-positive capillary endothelium. **C**, Human kidney: tubular epithelium and interstitial vessel with antigen-positive endothelial cell. Note nonspecific staining of renal tubular epithelial cells. **D** *Peromyscus maniculatus* lung: alveolus with antigen-positive capillary. **E**, *P. maniculatus* heart: atrial myocardium with antigen-positive endothelium. **F**, *P. maniculatus* kidney: tubule and glomerulus, no antigen detected. All tissues were stained with polyclonal murine serum directed against recombinant SNV–nucleocapsid antigen protein. Arrows indicate positive antigen staining. ×1000.

clinical and pathologic findings observed in humans with HPS [14]. In addition, the lack of seroconversion of any of the remaining *P. maniculatus* in the colony would suggest that hantavirus infection is not readily or rapidly passed among cohabitating positive and negative animals [15]. Our finding that virus-positive animals with serum antibodies to viral protein have relatively few viral antigen–positive endothelial cells may reflect a low level of viral replication in these animals. This is consistent with both the low transmissibility of the virus in rodent colonies as well as the lack of associated disease in chronically infected *P. maniculatus*.

Patients infected with SNV undergo an acute, rapid disease course. Their tissues contain abundant viral antigen in vascular endothelial cells, especially in the lung. This finding suggests that at the time of death, they had a relatively high virus burden. However, there have also been reports of people with serum antibody against SNV but only mild disease; interestingly, it is often more difficult to detect SNV RNA in the blood of these patients by RT-PCR (Hjelle B, unpublished results). Further studies are necessary to determine the role of virus burden on the clinical expression of HPS and virus tissue localization during acute through chronic

infection and the role of host factors that may contribute to this process.

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Analysis of the Persistence of Humoral and Cellular Immunity in Children and Adults Immunized with Varicella Vaccine

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The persistence of humoral and cellular immunity to varicella-zoster virus (VZV) was evaluated in 60 children and 18 adults immunized with live attenuated VZV vaccine. At a mean of 5 years after vaccination, 93% of children and 94% of adults had IgG antibodies to VZV as determined by ELISA. VZV antibody concentrations were significantly higher at 5 years than at 1 year after immunization in children and adults. Cell-mediated immunity to VZV was detected in 87% of children and 94% of adults at 5 years. The mean stimulation index was significantly higher at 5 years than at 1 year among children and adults. Cytokine responses to VZV, including interleukin-2, interferon-γ, and interleukin-10 were equivalent between children and adults at 5 years. In summary, varicella immunization induced long-term humoral and cellular immunity, and initial differences between cell-mediated responses in children and adults diminished over time.

Immunization with the live attenuated varicella vaccine elicits protection against varicella-zoster virus (VZV), a human herpesvirus that causes varicella (chickenpox) and reactivates as herpes zoster (shingles) [1]. The varicella vaccine was developed and evaluated in clinical trials in Japan in the 1970s [2]. In the United States, clinical trials were conducted in children with leukemia and later in healthy children [3]. The varicella vaccine elicits humoral and cellular immunity in children and adults [4, 5]. Early vaccine studies demonstrated an age-related difference in seroconversion rates, resulting in the recommendation of a two-dose regimen for adolescents and adults [6, 7]. Whether the initial administration of the varicella vaccine induces prolonged immunity or whether revaccination will be needed is an important question.

The vaccine, produced by Merck (West Point, PA), is made from the Oka isolate of VZV; it is produced in human diploid cells and given as a subcutaneous inoculation. Clinical attenuation of this vaccine has been shown in pre- and postlicensure experience [2, 8, 9]. Our analysis of the virologic basis of attenuation demonstrates that it results from impaired replication in epidermal and dermal cells [10].

The host response to primary infection with VZV involves synchronous induction of humoral and cell-mediated immune responses, which are important for the resolution of VZV infection and maintenance of latency [1]. Natural immunity is characterized by the long-term persistence of circulating T cells that recognize VZV proteins and by the persistence of IgG antibodies to VZV.

After natural infection, cellular immunity to VZV, measured as in vitro T cell proliferation, is well-preserved until the fifth decade of life or later. An age-related decline in cellular immunity to VZV then occurs, which predisposes to herpes zoster. VZV-specific T lymphocytes are found in $CD4^+$ and $CD8^+$ subsets. Within the $CD4^+$ subset, T cells that recognize VZV protein are predominately of the Th1 $CD4^+$ subset, producing interleukin (IL)-2 and interferon (IFN)-γ [11]. Cytokine profiles of elderly immune persons demonstrate that Th1 type $CD4^+$ T cell function declines significantly with age, whereas Th2 type responses, detected by IL-4 production, do not [11]. Thus, induction and preservation of Th1 $CD4^+$ T cell immunity appears to represent the optimal response to VZV.

The purpose of this study was to assess the long-term persistence of cellular immunity compared with that of VZV IgG antibodies in immunized children and adults and to characterize the relative preservation of Th1 and Th2 type cytokine responses.

Patients, Materials, and Methods

Study populations. Studies of the immunogenicity of the Oka-Merck varicella vaccine were done at Stanford University between 1988 and 1992 [4, 5]. The population in the current study consisted of vaccinees who seroconverted and did not develop breakthrough varicella.

Two hundred healthy children (ages 2–12 years), who were vaccinated between 1988 and 1990, received one dose of 1140–5850 pfu of infectious virus or two doses of 3315, 3625, or 9000 pfu. Sixty (30%) of these vaccinees agreed to be reevaluated [5].

Forty healthy adolescents and adults (ages 13–45 years), who were vaccinated between 1990 and 1992, were given two doses

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Informed consent was obtained from subjects prior to blood sample collection. The research conformed to the guidelines established by the Administrative Panel on Human Subjects in Medical Research at Stanford University, in accordance with federal regulations.

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of vaccine (905–9000 pfu/dose) [5]. Eighteen of these vaccinees (45%) agreed to be reevaluated.

Twenty-six (13%) of 200 children and 1 (2.5%) of 40 adults were excluded due to breakthrough varicella.

Humoral immunity. Serum was collected at the time of vaccination, 3 months after vaccination, 1 year after vaccination, and at follow-up. The presence of anti-VZV IgG antibodies was measured by ELISA to unfractionated VZV-infected cell lysates (antigen) and uninfected cell lysates (control) as previously described [12]. Initial sera were retested in parallel with follow-up sera to eliminate assay-to-assay variability.

VZV-specific lymphocyte proliferation assay. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood and incubated with unfractionated VZV-infected cell lysates or an uninfected cell lysate using methods previously described [5]. T cell recognition of VZV antigen was determined by the stimulation index (SI), which is the ratio of mean counts per minute in antigen-stimulated wells to that in control-stimulated wells. A positive response was defined as an SI of ≥ 3.0. The method used to prepare antigen batches has been standardized to minimize variability between preparations; the antigenicity of each preparation is evaluated using naturally immune subjects.

Assays for cytokine production. Cytokine release by PBMC was evaluated by stimulation with VZV antigen or uninfected cell control; maximum release in supernatants harvested on days 1–8 was determined. IL-2 was detected using kits from Genzyme (Cambridge, MA); IL-10 and IFN-γ were detected using kits from Endogen (Cambridge, MA). Cytokine concentrations were determined using a series of standards and extrapolated from a standard curve. Sensitivities of detection were defined by reference standards in each assay.

Exposure surveys. Five (8.3%) of 60 children and 1 (6%) of 18 adults reported a household exposure to VZV since vaccination. Nine children (15%) and 3 (17%) adults reported a nonhousehold, close-contact exposure that lasted >4 h.

Statistical analysis. Statistical analyses were done using a computer software program (Microsoft Excel, version 5.0; Microsoft, Redmond, WA). Comparisons were made using Student's paired and unpaired *t* tests.

Results

Study population. 60 children and 18 adults were reevaluated in our follow-up study. Fifteen of 60 children (25%) and all adults received two doses of vaccine. For children, the mean interval after vaccination was 6.4 years (range, 2.1–8.4) and for adults was 4.7 years (range, 3.6–6.2). The mean age at vaccination was 5 years (range, 2–12) for children and 34 years (range, 23–43) for adults.

VZV IgG antibody responses. Three-month seropositivity rates were 100% for children and adults; 1-year seropositivity rates were 97.7% (43/44) for children and 100% (14/14) for adults. At 3 months, adults and children who received two doses of vaccine had significantly more VZV antibody than children who received one dose of vaccine (optical density ± SE: in adults, 1.06 ± 0.16; in children after two doses, 1.03 ± 0.15; in children after one dose, 0.55 ± 0.11; $P < .05$). Five years after vaccination, seropositivity rates were 93.3% (56/60) for children and 94.4% (17/18) for adults. An increase in antibody concentrations from 1 to 5 years was significant for both adults and children ($P < .05$). Antibody levels were equivalent between adults and children at 1- and 5-year time points (figure 1A).

VZV-specific T lymphocyte proliferation. One year after vaccination, 89.7% (26/29) of children and 94.1% (16/17) of adults had cellular immunity to VZV. At 5 years, VZV-specific T cell proliferation was maintained in 86.7% (52/60) of children and 94.1% (16/17) of adults. The mean SI ± SE at 1 year was 12.08 ± 2.03 for children and 9.89 ± 1.80 for adults, compared with a mean at 5 years of 22.08 ± 2.35 in children and 22.39 ± 4.78 in adults. Responses of children and adults were equivalent at 1 and 5 years.

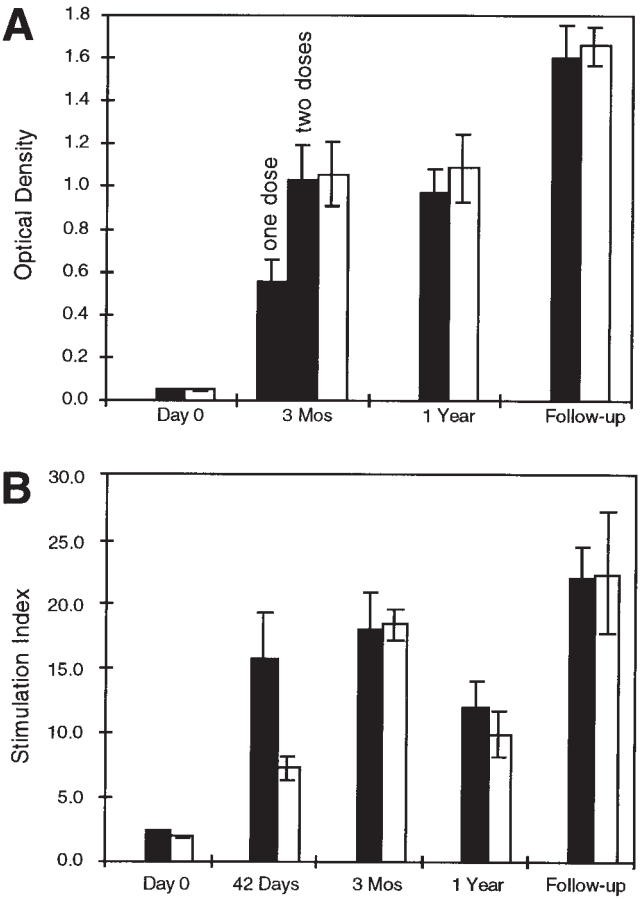

Figure 1. **A**, Persistence of IgG antibodies to varicella-zoster virus (VZV). There is significant increase in 3-month antibody levels in children (black bars) and adults (white bars) who received 2 doses of vaccine, compared with levels in children who received 1 dose ($P < .05$). Difference between responses at 1 year and follow-up is significant ($P < .05$) for children and adults. **B**, Persistence of T cell recognition of VZV. Stimulation indices (SI) <3.0 are considered negative. Mean SI responses were increased significantly at follow-up vs. 1 year in children (black bars) and adults (white bars) ($P < .05$). Mos = months.

The mean SI ± SE at day 42 was significantly greater in children than in adults (15.59 ± 3.67 vs. 7.19 ± 0.95; $P = .03$). The mean SI was equivalent at 3 months, by which time, all adults had received two doses of vaccine. Responses diminished in both groups between 3 months and 1 year after vaccination ($P = .13$ and .02 for children and adults, respectively). The SIs in individual vaccinees increased significantly between 1 year and the follow-up specimen ($P = .04$ for both children and adults) (figure 1B).

No statistically significant association between the infectious virus content of the vaccine and the persistence of T cell recognition of VZV antigen was demonstrated.

Cytokine profiles. Cytokine release profiles of proliferating T cells were evaluated when follow-up blood sampling was done. The value in picograms per milliliter was calculated as the difference between the response to the VZV antigen and control. Samples were considered positive if the level of detectable cytokine was above the sensitivity of detection defined by reference standards for each assay. Analysis of the kinetics of cytokine release from days 1 through 8 revealed no age-related differences; peak responses for IL-2 occurred at 2–4 days, and those for IL-10 and IFN-γ occurred at 5–7 days. The interval-to-peak response was equivalent in children and adults.

IL-2 was detected in 71% (36/51) of PBMC cultures from children and 50% (8/16) of cultures from adults. The mean IL-2 concentration in positive cultures was 496.0 ± 53.6 in children and 511.7 ± 87.7 in adults. IFN-γ was detected in 91% (43/47) of PBMC cultures from children and 93% (14/15) from adults. The mean value for IFN-γ in positive cultures was 685.4 ± 78.2 in children and 737.7 ± 163.6 in adults. IL-10 values were 214.2 ± 38.2 in children and 200.8 ± 44.0 in adults. IL-10 was at detectable levels in supernatants of PBMC cultures from 60% (30/50) of children and 38% (6/16) of adults (figure 2). Background levels (mean concentrations in control-stimulated wells) are given in figure 2. The concentrations of cytokines produced in PBMC cultures for children and adults were equivalent and did not correlate with the SI.

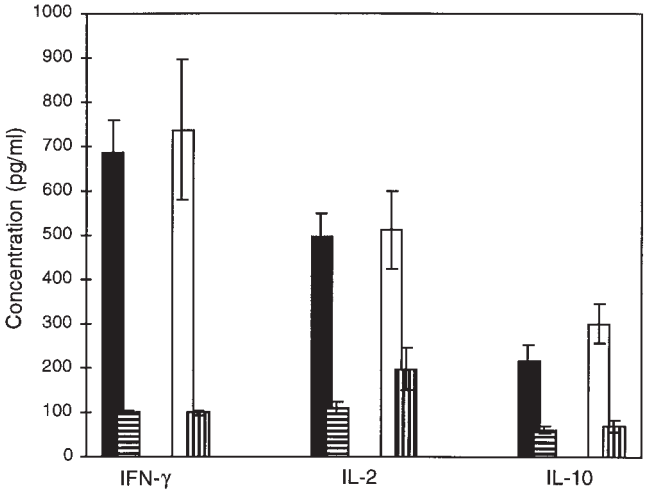

Figure 2. Cytokine release from varicella-zoster virus (VZV)–stimulated peripheral blood mononuclear cells. Bars indicate mean concentration in VZV antigen–stimulated wells for children (black) and adults (white) and in control-stimulated wells for children (horizontally striped) and adults (vertically striped). IFN = interferon, IL = interleukin.

Discussion

In our study, antibodies to VZV were well-preserved at a mean of 5 years following vaccination in adults and children. Similarly high rates of antibody persistence have been observed in healthy children; surveillance studies have demonstrated that the vaccine induces antibody responses that persist for at least 20 years [2, 8]. Lower VZV IgG titers were associated with lower cellular immune responses in adult vaccinees [5]. However, with longer follow-up, VZV-specific IgG antibody concentrations increased significantly in adults and children. The increase in antibody titers with time after immunization has been observed by others [13]. Our study demonstrated that even among vaccinees with low initial responses, the antibody concentrations can be expected to increase over time and that the two-dose vaccine regimen is sufficient to achieve long-term equivalence of humoral immunity to VZV in adults and children.

The capacity to elicit cell-mediated responses is an important factor, accounting for the long-term protective efficacy of live attenuated viral vaccines. From 98% to 100% of healthy children given the varicella vaccine have T cell recognition of VZV antigens by 2–6 weeks after immunization with one dose; however, the initial T cell responses of adult vaccinees are lower [5]. In our experience with longer follow-up, cell-mediated immunity was found to increase significantly in children and adults, with the responses of adults becoming equivalent to those of children with vaccine-induced immunity.

Consistent with the significant role of the cell-mediated immune response to varicella, VZV infection elicits the proliferation of T cells that produce predominantly Th1 type cytokines [4]. A prominent IFN-γ response is characteristic of naturally acquired immunity to VZV [11]. In our follow-up study, the protection elicited by immunization with live attenuated varicella vaccine was characterized by antigen-specific IL-2 and IFN-γ release by activated T cells. No age-related differences in the cytokine secretion patterns of cultured T cells were detected with long-term follow-up in children and adults who had vaccine-induced immunity.

Surveillance studies indicate that breakthrough varicella occurs at an incidence of about 2%–3% per year in children and adults but that the incidence and severity do not increase over time [2, 14]. Our finding that immune responses to VZV are preserved well after varicella vaccination is consistent with these clinical observations.

Whether the immunologic basis for the increase of VZV immune responses in vaccinees was due to endogenous or ex-

ogenous reexposure could not be determined because the annual epidemics of varicella continued to occur during the study interval. As more of the population receives the varicella vaccine, the opportunities for exogenous exposure to wild-type VZV will decrease. If these exposures are the major mechanism for preserving high levels of immunity to VZV, it may be necessary to provide additional booster doses of varicella vaccine. Watson et al. [15] demonstrated that a booster dose of varicella vaccine given 4–6 years after the initial dose boosts both humoral and cellular immunity. Alternatively, since the immunologic basis for long-term immunity to viral pathogens in general has not been established, continued evaluation of varicella vaccinees may demonstrate that restimulation is not required to maintain memory T cell responses to VZV that sustain B cell and effector T cell populations.

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Spontaneous Loss of Viral Episomes Accompanying Epstein-Barr Virus Reactivation in a Burkitt's Lymphoma Cell Line

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Life-long viral persistence is a hallmark of human herpesvirus infection. In the Epstein-Barr virus (EBV)–positive Burkitt's lymphoma (BL) cell line, Mutu, spontaneous loss of all viral episomes accompanied productive viral DNA replication. The molecular configuration of intracellular EBV DNA evolved from monoclonal episomes in cells retaining the original tumor phenotype to predominantly replicating linear DNA and, subsequently, only integrated forms in BL cells that had acquired the lymphoblastoid cell phenotype. Transient appearance of deleted, rearranged WZhet EBV DNA capable of disrupting viral latency, along with the integration of viral DNA into human chromosomes, indicates a genetic instability in the host cell which, if duplicated in vivo, may affect configuration and persistence of the viral genome in expanding malignant cell clones.

Burkitt's lymphoma (BL) is a B cell neoplasm that occurs in children and young adults worldwide. Epstein-Barr virus (EBV) is variably associated with the tumor, being found in virtually all cases of African BL, half of South American BL, and ~20% of cases in the United States [reviewed in 1]. In EBV-linked disease, viral initiation of tumorigenesis is suggested by molecular evidence for the presence of EBV DNA in a single tumor progenitor cell prior to its malignant conversion [2, 3]. Infection precedes and therefore is likely to contribute to clonal expansion.

Within latently infected cells, the EBV genome is maintained in multiple episomal copies. Cultured, EBV-positive BL cell lines generally contain from <10 to as many as 100 episomes per cell. While varying among cell lines, the viral copy number remains largely constant over time for each individual cell line. In the African BL line Mutu [4], we investigated the complete loss of episomal DNA observed during long-term culture. Such spontaneous viral DNA loss mimics aspects of unstable maintenance of EBV DNA in tumors in vivo [5, 6] and may be relevant to the less than total association of EBV with human malignancies that share clinical, histopathologic, and cytogenetic features.

Materials and Methods

Cell lines and EBV expression. The BL-derived cell line Mutu, clone 59 (group I/Burkitt's-like) and clone 176 (group III/LCL-like) (gift of A. B. Rickinson, University of Birmingham, United Kingdom) [4] were maintained in RPMI 1640 with 10% fetal bovine serum. Relatedness of each Mutu clone after long-term culture was confirmed by analysis of cell karyotype (St. Jude Children's Cancer Center Cytogenetics Facility) and immunoglobulin gene rearrangements using a probe derived from a 3.4-kb pair (kbp) *Eco*RI/*Hin*dIII fragment of the joining region (J_H). Stability of the phenotypic grouping of each clone [4] was assessed by standard immunoblotting for EBV protein expression using human serum from a patient with nasopharyngeal carcinoma and by indirect immunofluorescent staining of cells using monoclonal antibodies PE2, CS1-4, and BZ.1 (gift of L. S. Young, University of Birmingham, United Kingdom) specific for EBV proteins EBNA2, LMP1, and BZLF1, respectively.

Southern analysis of EBV DNA. The molecular configuration of EBV termini (linear, episomal, or integrated) was determined by the method of Raab-Traub and Flynn [2], using ^{32}P-labeled riboprobes specific for regions of unique EBV DNA (*Xho*I-a and *Bam*HI-J portion of *Eco*RI-I; gift of N. Raab-Traub, University of North Carolina) immediately internal to terminal repeat sequences at either end of the viral genome. Total cellular DNA from BL cell lines was digested with *Bam*HI restriction endonuclease, loaded at 10 μg of DNA per well, separated by electrophoresis in 0.8% agarose gels, and then transferred to nylon membranes for hybridization.

Fluorescent in situ hybridization. The cosmid cM302-21 [7], containing >40 kbp of EBV DNA, was used to create probes labeled by nick-translation with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis). Probe was hybridized to fixed interphase or metaphase chromosomes as previously described [8]. Hybridized probe was detected by fluorescein-conjugated sheep antibodies to digoxigenin (Boehringer Mannheim).

Detection of WZhet DNA by polymerase chain reaction (PCR). Twenty-five cycles of PCR amplification were performed on 100 ng DNA with Taq polymerase using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) as per manufacturer's recommendations. Primers were selected that framed the junction of rearranged DNA in WZhet EBV (5′-TGGGACGTGCTAAATTTAG-3′ and 5′-GTCCAGCGCGTTTACGTAAG-3′). Probes specific to both

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of the abnormally juxtaposed W and Z *Bam*HI fragments were used to detect the predicted 301-bp PCR product [6]. P_3HR_1 clone 5 (from G. Miller, Yale University, New Haven, CT), which contains WZhet DNA, served as positive control.

Results

On serial passage, BL cells typically drift from the original biopsy cell phenotype (termed group I: CD10/CD77-positive, activation antigen/adhesion molecule–negative) expressing only one latent viral nuclear antigen (EBNA1) to a lymphoblastoid cell line phenotype (termed group III: CD10/CD77-negative, activation antigen/adhesion molecule–positive) in which cells express all nine latency-associated EBV proteins and some enter the EBV lytic cycle [4, 9]. Cell clones of Mutu BL have been established that grow stably in either the group I or III phenotype [4]. After ~30 months in culture, Mutu group III cells yielded a progressively diminished hybridization signal for EBV DNA on standard Southern analysis (not shown).

Because group III cells had previously been described as containing significant numbers of cells in lytic cycle [4], the observed change in viral DNA content might merely reflect cessation of EBV replication in that subset of cells. To assess this possibility, we analyzed DNA from early and late passages of Mutu group I and group III culture phenotypes for the configuration of EBV termini (figure 1). *Bam*HI restriction endonuclease digestion of viral DNA preserves terminal repeat sequences that vary in number for each virion but cleaves adjacent unique DNA. Fused termini, indicated by a high-molecular-weight band that hybridizes to probes specific for opposite ends of the genome, denote circular forms found in latently infected cells, whereas an array of smaller unjoined ends varying in size by the ~500 bp in each repeat represents linear viral DNA characteristic of replicative infection [2].

As shown in figure 1 (blots A and B), both the early- and late-passage Mutu cells retaining the BL group I phenotype contained predominantly episomal EBV DNA. Detection of a single high-molecular-weight fragment with probes specific for unique sequences at either end of the EBV genome confirm that the BL line contained a single clonal population of episomal DNA. A minor component of replicating virus was detected in the late passage, as represented by small-molecular-weight unjoined termini. By contrast, exuberant replication was seen in the early-passage group III clone in the presence of a persisting, yet proportionally diminished, episomal DNA population (blot C). As was anticipated, late-passage group III cells now contained no evidence of replicating virus, accounting in part for the reduction in EBV DNA content observed. Surprisingly, however, the single 20-kb band representing joined EBV termini in both group I and early-passage group III Mutu cells was also absent (blot D). Instead, the episomal band was replaced by two high-molecular-weight bands, the sizes of which varied when probes to the right versus left end of the EBV genome were used. These results suggested the EBV termini were no longer joined as if in the circular configuration but integrated with cellular sequences of varying length, the two bands identified by each probe representing individual integration events.

To corroborate evidence for viral integration, interphase and metaphase chromosomes were examined by fluorescent in situ hybridization, using as probe EBV DNA from a large cosmid clone covering approximately a quarter of the viral genome [7]. Consistent with the termini analysis, metaphase spreads from multiple mitoses repeatedly demonstrated symmetrical doublet signals on sister chromatids at two chromosomal sites, indicating EBV DNA integration (figure 1). On the basis of the possibility that the spontaneous changes observed in EBV DNA configuration in this late-passaged cell population represented inadvertent contamination of cell lines, we analyzed both the karyotype and immunoglobulin gene rearrangements in Mutu III early and late passages, each of which were identical for the two cell populations (data not shown).

EBV replication in another BL-derived cell line, P_3HR_1, has been directly linked to presence of defective, rearranged EBV DNA, termed heterogeneous (or WZhet) DNA [10]. Since EBV integration may reflect a more pervasive DNA instability that might foster both defective virus and episomal loss, we used PCR analysis to examine Mutu total cellular DNA for WZhet rearrangements (figure 2). PCR primers that flanked the aberrant junction between two EBV DNA fragments, *Bam*HI-W and -Z, contiguous in WZhet DNA but 55 kbp apart in the standard genome, yielded a product in the same Mutu group I and group III populations that by Southern analysis (figure 1) contained replicating virus. PCR products were somewhat larger from Mutu than from the P_3HR_1 control, suggesting variability in the junction between rearranged fragments. However, each product hybridized to oligonucleotide probes internal to primer sequences and specific for either the *Bam*HI Z or W fragment (figure 2).

Because the BZLF1 (*Bam*HI-Z leftward open-reading frame 1) protein encoded by both standard and defective genomes initiates the switch from EBV latency to virus replication [10], late-passage group I and group III clones that differed with regard to state of viral activation (figure 1, blots B and D) were stained by indirect immunofluorescence for BZLF1. Consistent with DNA analyses that demonstrated the presence of WZhet DNA and viral replication, late-passage group I cells contained a subset of BZLF1-positive cells in contrast to late-passage group III cells (figure 2), a pattern just the reverse of that originally described for early Mutu cell passages [4]. The pattern of viral latent gene expression definitive for group I and group III cells, however, remained unchanged (not shown).

Discussion

The notion that viruses may be responsible for genetic damage, yet fail to persist in the malignant cell, has previously been advanced in the case of those human herpesviruses (e.g.,

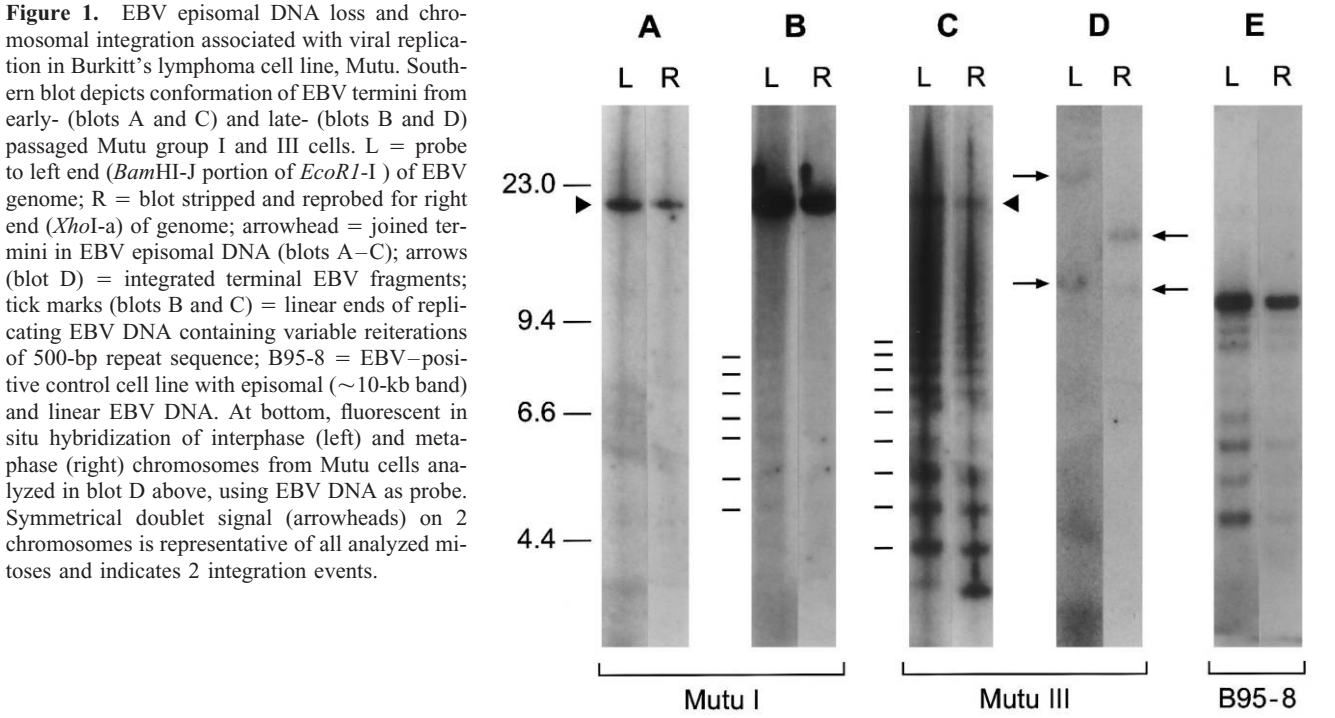
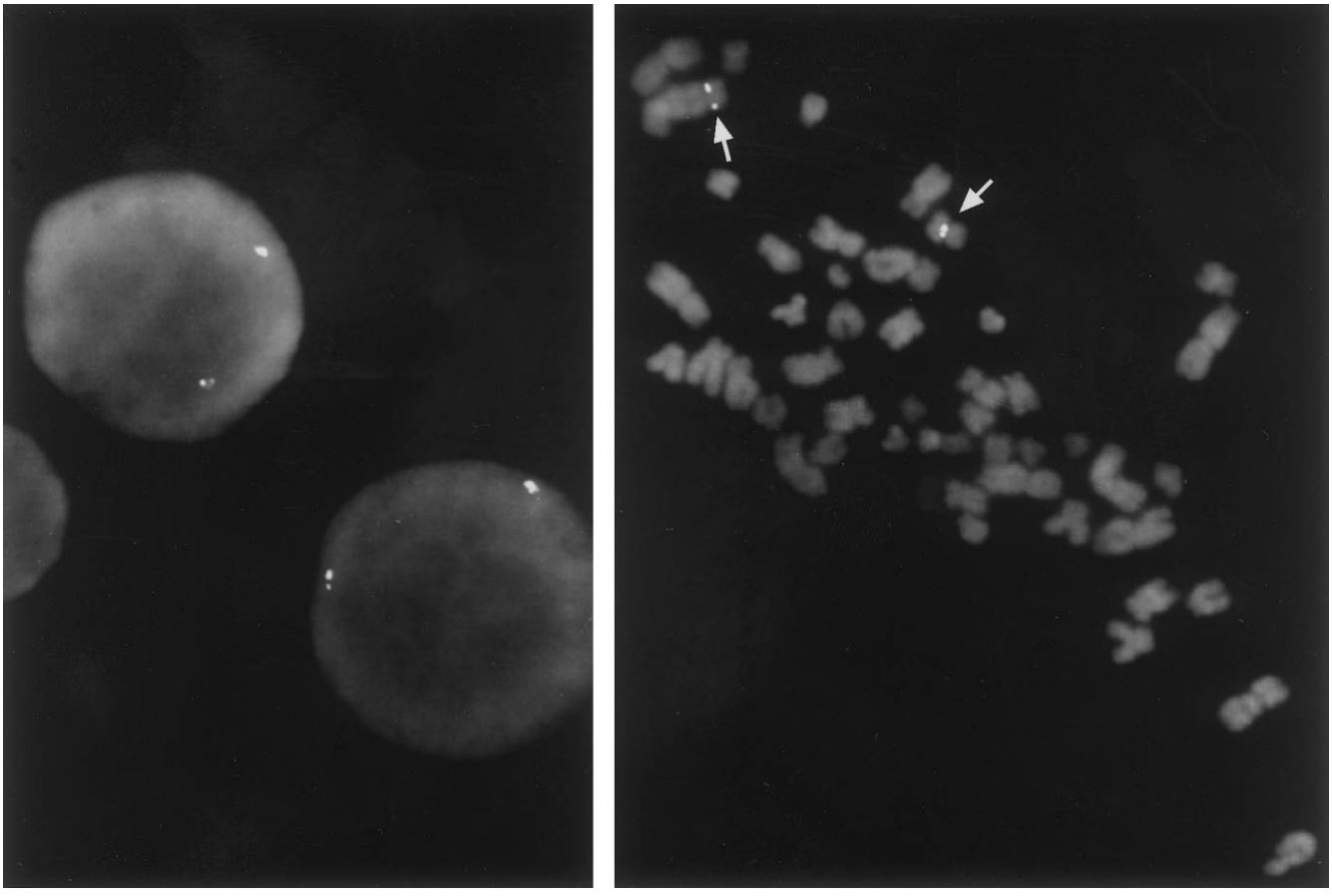


Figure 1. EBV episomal DNA loss and chromosomal integration associated with viral replication in Burkitt's lymphoma cell line, Mutu. Southern blot depicts conformation of EBV termini from early- (blots A and C) and late- (blots B and D) passaged Mutu group I and III cells. L = probe to left end (*Bam*HI-J portion of *EcoR1*-I) of EBV genome; R = blot stripped and reprobed for right end (*Xho*I-a) of genome; arrowhead = joined termini in EBV episomal DNA (blots A–C); arrows (blot D) = integrated terminal EBV fragments; tick marks (blots B and C) = linear ends of replicating EBV DNA containing variable reiterations of 500-bp repeat sequence; B95-8 = EBV–positive control cell line with episomal (~10-kb band) and linear EBV DNA. At bottom, fluorescent in situ hybridization of interphase (left) and metaphase (right) chromosomes from Mutu cells analyzed in blot D above, using EBV DNA as probe. Symmetrical doublet signal (arrowheads) on 2 chromosomes is representative of all analyzed mitoses and indicates 2 integration events.



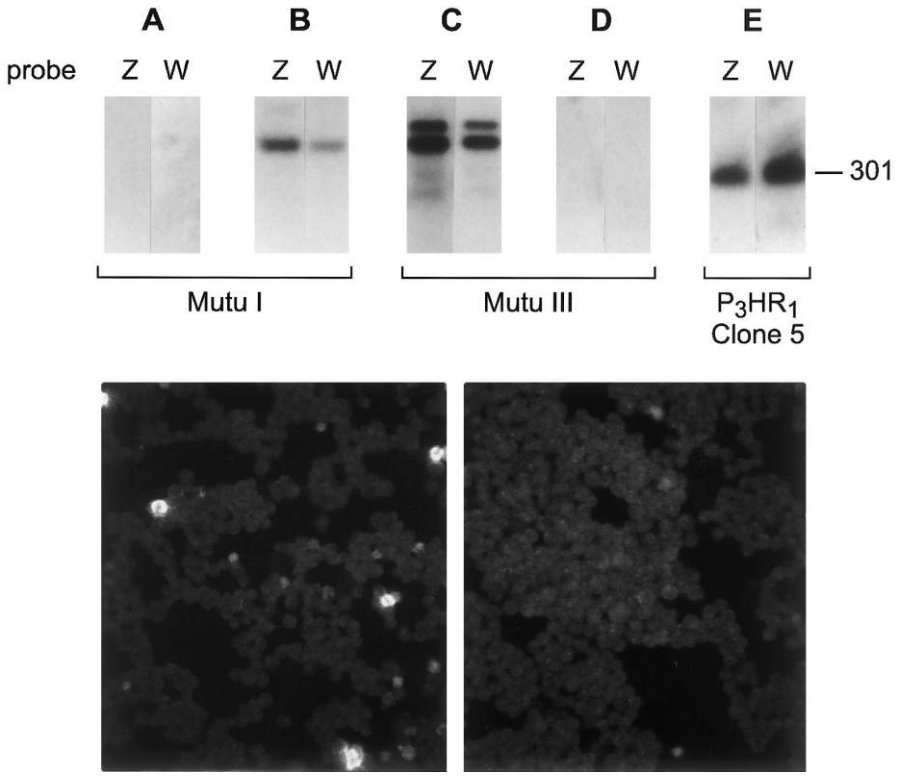


Figure 2. Rearranged WZhet EBV DNA in cell populations replicating EBV. Using total Mutu DNA (early [blots A and C] vs. late [blots B and D] cell passages), polymerase chain reaction products were derived from sequences spanning junction of abnormally juxtaposed *Bam*HI-W and -Z EBV DNA fragments. P_3HR_1 clone 5 = WZhet-positive control; expected product size is 301 bp. At bottom, late-passage Mutu cells analyzed in blots B (left) and D (right) above were stained by indirect immunofluorescence for EBV immediate-early (replicative cycle) protein BZLF1 with monoclonal antibody BZ.1.

herpes simplex virus) for which little positive evidence exists to support an association with human cancer [11]. Conceptually, such a ''hit-and-run'' mechanism of viral oncogenesis might be more convincingly argued for EBV, which encodes known oncoproteins but whose well-documented associations with the human malignancies Hodgkin's lymphoma and BL are less than complete. Just such a process of viral DNA loss has been recently suggested by findings of heterogeneity within a single BL in the form of EBV-positive and EBV-negative cells bearing identical chromosomal abnormalities [5].

Our findings of complete episomal DNA loss, accompanied by viral DNA integration, complement an earlier report of EBV DNA loss from the BL cell line Akata, where up to half of the cells failed to maintain the viral episomes after 2 years in culture [12]. Although affected Mutu cells retained their group III phenotype, including expression of all latent EBV proteins, viral integration presumably offered some selective advantage leading to the outgrowth of this subpopulation. Alternatively, Mutu cell variants with integrated EBV inapparent on initial DNA blots may have preexisted EBV lytic replication and have been selected for during cytolysis. Episomal and integrated copies of EBV have been known to coexist in some BL cell lines [13], but marked episomal EBV DNA loss with clonal emergence of a population containing integrated virus has not been described. What makes these observations of particular note is that the footprints of viral instability described here for Mutu (EBV reactivation, WZhet EBV DNA rearrangement, episomal DNA loss, EBV DNA integration) closely parallel findings reported in uncultured sporadic BL tissue biopsies in which presence of only integrated EBV DNA or rearranged WZhet DNA led to their misclassification as EBV-negative tumors [6]. As indicated in that report, integrated viral DNA may not contribute to the malignant process but rather attests to the overall genomic instability within the cell.

The clearance of viral episomes from a population of cells also containing WZhet EBV DNA may be more than coincidental. The rearrangement of *Bam*HI-W and *Bam*HI-Z EBV fragments confers unusual biologic properties on the defective DNA as a result of constitutive expression of the immediate-early gene BZLF1 [14], transient transfections of which have been shown to cause partial elimination of EBV episomes from BL cells [15]. Since WZhet DNA itself is not stably associated with cells [10], both might be effectively lost from an expanding cell clone. Unless episomal loss was accompanied by EBV DNA integration, evidence for past infection of tumor would be absent.

The consistency with which t(8:14), t(2:8), and t(8:22) translocations are found in all BL makes the juxtaposition of *c-myc* and immunoglobulin gene loci a critical step in lymphomagenesis. Agreement on the contribution of virus has been more problematic due to the only partial association of EBV with BL, an issue that can be resolved if we accept a priori that transforming events other than EBV infection can lead to the same histological and cytogenetic abnormalities. Our data raise

yet another possibility that may be operational in a subset of BL. EBV initiation of tumorigenesis may be followed by loss of all or part of the viral genome early in clonal expansion.

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Stability over Time of Serum Antibody Levels to Human Papillomavirus Type 16

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The stability over time of serum IgG antibody levels to human papillomavirus type 16 (HPV-16) was determined by comparing the HPV-16 capsid antibody levels in serial serum samples of an age-stratified random subsample of 1656 primiparous mothers resident in Helsinki who were followed until their second pregnancy, on average 29.5 months later. The correlation between the first and second pregnancy HPV-16 serum antibody levels of the same woman was high, even when >4 years had elapsed between pregnancies ($r = .822$). Between negativity, indeterminate results, or quartiles of positivity, the predictive values for being classified in the same category on both occasions ranged between 42% and 91%. Correlation coefficients, predictive values, and κ coefficients between serial samples all were comparable with those of repeat analyses of the same sample, indicating that HPV capsid antibody levels are generally stable during several years of follow-up.

The oncogenic genital human papillomaviruses (HPVs) are widespread sexually transmitted agents that are established (especially HPV-16) as a major cause of anogenital cancers [1]. Historically, HPV infection has been assayed by detection of viral DNA in cell samples. However, a single test for the detection of HPV DNA, no matter how sensitive, may be inaccurate because of sampling errors [1, 2]. Also, in follow-up studies of HPV DNA-positive women, a majority have cleared their HPV DNA within 1–2 years [1]. Thus, the only way to detect previous HPV infections, that is, cumulative exposure, or an infection at a site not accessible to sampling is by detecting an antibody response. HPV seroepidemiology is therefore important for the continuing elucidation of the natural history of HPV infection, its role in malignancies, and the spread of the infection in different populations.

In general, interpretable seroepidemiology requires knowledge of several indices of antibody responses and assay characteristics, the most important of which are sensitivity, specificity, biologic stability of antibodies over time, and assay variability.

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For the capsid-based HPV-16 serology, estimates of sensitivity for detecting HPV-16 exposure (using detection of HPV-16 DNA in cervical samples as reference) have ranged from 14% [3] to >80% [4, 5], with most studies reporting ~50% sensitivity [6–9]. Specificity for the sexually transmitted HPV types seems to be high, since no virginal and few monogamous women are seropositive [4, 7]. Seropositivity has not been associated with number of recent sex partners but associates very strongly with the lifetime number of sex partners (linear increase of seroprevalences with ~4%/partner) [7], arguing that seropositivity is rather a measure of lifetime cumulative exposure than of recent exposure. Seroprevalences have also not shown any consistent association with age [6, 7, 10, 11], in spite of the fact that HPV DNA prevalences are strongly age-dependent [1].

Evaluation of the biologic stability of capsid antibody levels over time is important for understanding the immunobiology of HPV infection and for interpreting HPV seroepidemiology. We took advantage of the population-based Finnish serologic screening of pregnant women for a large-scale evaluation of assay consistency and biologic stability of antibody levels in serial samples taken from the same women.

Materials and Methods

The National Public Health Institute in Finland organizes a population-based serologic screening program for congenital infections at the first trimester of pregnancy. The serum samples from >98% of all pregnant women in the country are sent to the National Public Health Institute for serologic analyses and since 1983 have been stored at −20°C. The Finnish maternity cohort collection and storage system had by 1996 stored >1,000,000 serum samples registered on computerized files. We selected a random subsample

of 1656 primiparous women, stratified by age at first pregnancy (6 strata of 13–17, 18–19, 21, 23, 25, and 30 years of age) among women who were resident in the Helsinki metropolitan area, had been pregnant for the first time during 1983–1984 or 1990–1991, and had had a second pregnancy during a 5-year follow-up period.

The standard direct ELISA method [8] for detecting IgG antibodies specific for HPV-16 by use of baculovirus-expressed capsids comprising both the L1 and L2 proteins was used, with disrupted capsids of bovine papillomavirus as negative control, as described in detail elsewhere [12]. The assays included several internal standard samples on each ELISA plate, both positive controls (pools of serum from patients with cervical dysplasia or cancer and from healthy women with cervical HPV-16 DNA) and negative control sera (sera from sexually inexperienced women). The optical density (OD) values from each plate were normalized relative to the results of the internal standard sera. The cutoff level was preassigned (OD of 0.100) and had previously been shown to distinguish HPV-infected from virginal women [4]. The intraassay coefficients of variation varied between 9.4% and 10.7% in different assay runs, and the interassay coefficients of variation varied between 9.9% and 23.9%.

The predictive value equals the number of subjects who stay in a percentile category during follow-up divided by the total number of subjects in that category at the initial measurement. The κ statistic quantifies the extent of tracking between percentile categories, beyond the amount of tracking expected by chance alone: $\kappa = [p(A) - p(E)]/[1 - p(E)]$, where $p(A)$ is the actual tracking of the group and $p(E)$ is the amount of tracking expected if individuals are randomly assigned to the different groups.

The Pearson's product-moment correlation coefficient (r) quantifies the degree of correlation between measurements.

Results

On the basis of the OD values of the 387 positive sera (23%) among the first pregnancy samples, 4 quartile groups with an equal number of subjects were defined (table 1). In addition, a similarly sized group of women with OD values just below the cutoff level were defined as an indeterminate group. The remaining 1169 women constituted the seronegative group.

As seen in table 1, the predictive values for the probability that the antibody levels of the second pregnancy samples of the same women tracked in the same group as at first pregnancy ranged between 91% and 42%. By comparison, a repeat analysis of 469 serum samples randomly chosen from the 6 groups found the predictive values to be of the same magnitude (ranging between 90% and 45%). The overall κ coefficient between the first and second pregnancy antibody level was .585 ($z = 40.8$; $P < .0001$). The antibody levels tracked well in the seronegative group and the group with the strongest level of positivity. Moderate tracking was observed for the other 3 quartile groups of positivity, whereas the indeterminate group tracked poorly. A similar tracking pattern was observed among the repeated measurements of the same sample.

The tracking between antibody level categories appeared to follow a Gaussian distribution, both for the serial pregnancy samples and for the repeated measurements, with most samples that moved between categories moving to the neighboring category (figure 1A, B).

The correlation between the antibody levels at first and second pregnancy was high and somewhat stronger for the women with a short (1–2 calendar years) lag between pregnancies than for women with a long (4–5 calendar years) lag (figure 1C, D). Storage time per se did not appear to affect antibody level variability, since the correlation coefficients between serial sample measurements were similar for women with their first

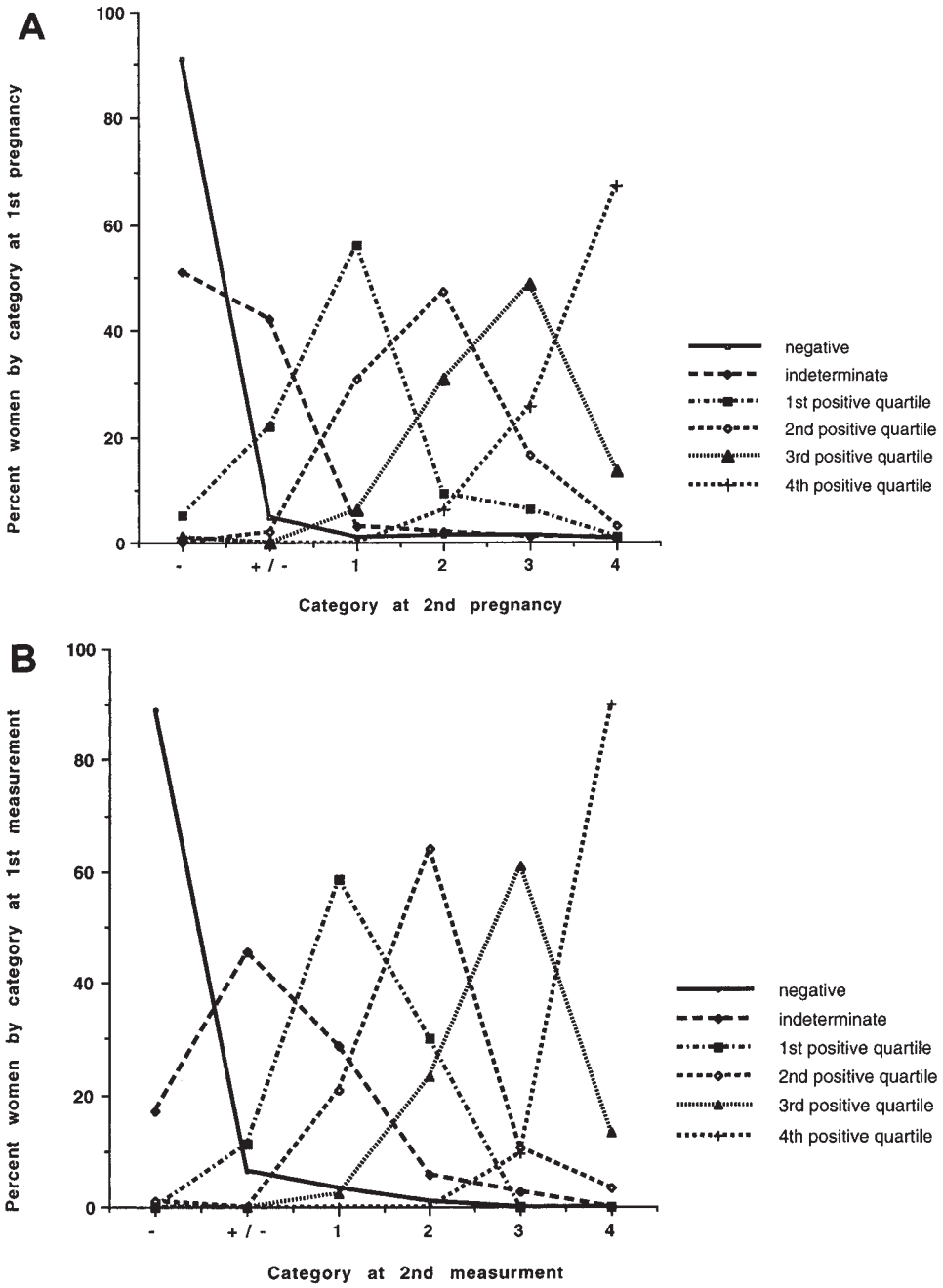
Table 1. Tracking of HPV serum antibody levels between pregnancies, in comparison with tracking of HPV antibody levels between repeat analyses of same serum sample.

Antibody level categories (dOD value limits*)	No. of women in category	Mean ± SD antibody level of 1st pregnancy sample (dOD × 1000)	Mean ± SD antibody level of 2nd pregnancy sample (dOD × 1000)	Predictive value between 1st & 2nd pregnancy sample (%)	κ coefficient between 1st & 2nd pregnancy sample	Predictive value between repeat measurements of same sample† (%)	κ coefficient between repeat measurements of same sample
Seronegative, 0–0.051	1169	11.56 ± 13.33	26.15 ± 70.25	91	.69	89	.86
Indeterminate, 0.052–0.099	100	70.79 ± 14.49	64.43 ± 69.33	42	.38	45	.40
1st quartile of positivity, 0.100–0.179	96	140.27 ± 24.11	144.13 ± 87.5	56	.53	59	.54
2nd quartile of positivity, 0.180–0.299	97	233.61 ± 36.97	236.95 ± 108.88	47	.44	64	.56
3rd quartile of positivity, 0.300–0.476	97	387.48 ± 52.55	358.77 ± 133.23	48.5	.45	59	.50
4th quartile of positivity, 0.477–1.208	97	640.29 ± 149.30	588.17 ± 208.27	67	.65	88	.84

NOTE. dOD, difference in optical density.

* dOD value limits were defined by first pregnancy/first measurement results. Same limits were used to categorize second pregnancy/second measurement results.

† Based on only 90, 35, 53, 84, 81, and 126 samples in each group, respectively.



pregnancy during 1983–1984 ($r = .875$, all women) and for women with their first pregnancy during 1990–1991 ($r = .858$, all women) (not shown).

The population of women who moved from absolutely seronegative at first pregnancy to clearly positive at second pregnancy (seroconverters) is seen in figure 1C, D (boxed areas), whereas a corresponding group of women moving from clearly positive at first pregnancy to negative at second pregnancy is not seen. Since seroconversions are a biologic phenomenon reflecting new infections, the correlation between antibody levels between first and second pregnancy was also calculated excluding the seroconverting women (figure 1C, D). The correlation coefficients were substantially increased and became comparable to the correlation coefficients found for repeat measurements of the same sample (figure 1E).

The rate of seroconversions between the two pregnancies was strongly associated with age at first pregnancy, increasing from 1.3% (when mothers are $\geq$30 years of age) to 18.8% (among mothers $<$18 years of age) (unpublished data). That very young mothers are at risk of sexually transmitted diseases

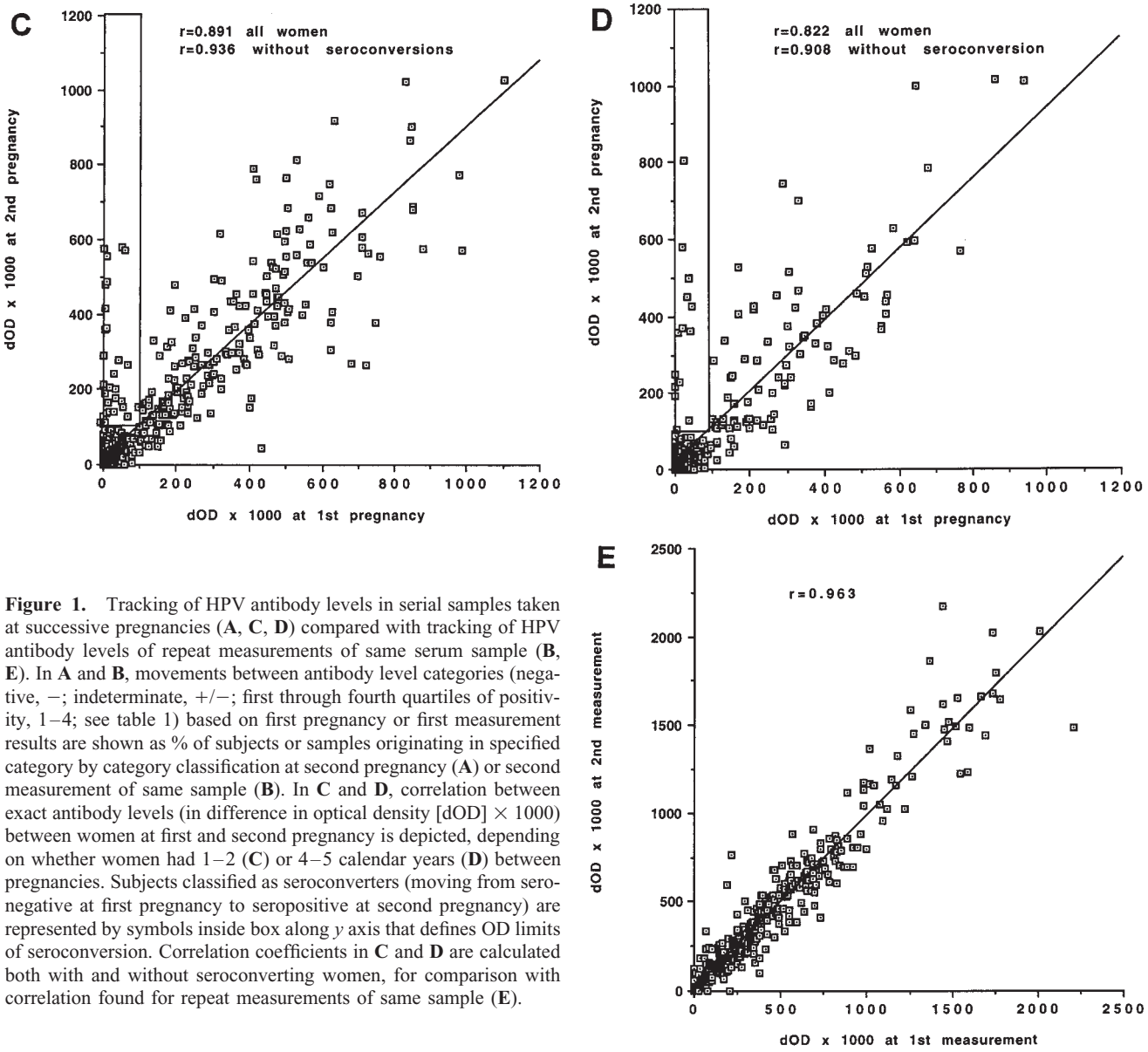


Figure 1. Tracking of HPV antibody levels in serial samples taken at successive pregnancies (**A**, **C**, **D**) compared with tracking of HPV antibody levels of repeat measurements of same serum sample (**B**, **E**). In **A** and **B**, movements between antibody level categories (negative, −; indeterminate, +/−; first through fourth quartiles of positivity, 1–4; see table 1) based on first pregnancy or first measurement results are shown as % of subjects or samples originating in specified category by category classification at second pregnancy (**A**) or second measurement of same sample (**B**). In **C** and **D**, correlation between exact antibody levels (in difference in optical density [dOD] × 1000) between women at first and second pregnancy is depicted, depending on whether women had 1–2 (**C**) or 4–5 calendar years (**D**) between pregnancies. Subjects classified as seroconverters (moving from seronegative at first pregnancy to seropositive at second pregnancy) are represented by symbols inside box along *y* axis that defines OD limits of seroconversion. Correlation coefficients in **C** and **D** are calculated both with and without seroconverting women, for comparison with correlation found for repeat measurements of same sample (**E**).

is well known in venereology, supporting that seroconversions are a biologic phenomenon reflecting new infections. The opposite phenomenon, seroreversion (i.e., moving from positive to negative), had no association with age at first pregnancy. Seroreversions were detected in 1.8% of the samples, but most of the seroreversions (90%) originated in the lowest quartile of positivity and 77% of these ''reverting'' women moved only to the indeterminate and not to the seronegative category, suggesting that a substantial amount of seroreversions are explained by misclassification (cf. figure 1A and 1B). In contrast, 88% of seroconversions originated in the seronegative group and most (74%) of these seroconversions moved to the 3 higher quartiles of positivity.

Only 2 women moved from the 3rd or 4th quartile group of positivity at first pregnancy down to seronegativity at second pregnancy (figure 1A). Since the movements of these women were clearly outliers from the Gaussian distribution of movements (figure 1A, B), the possibility of error should be considered. Laboratory error was excluded by multiple repeat analyses, but other possibilities of error exist (e.g., sampling of the wrong woman, wrong entry of serum identity or personal identification numbers, and incorrect conditions for specimen handling, transportation, or storage).

Discussion

The field of HPV epidemiology has provided one of the clearest practical examples of the devastating effect of misclassification in molecular epidemiology, in which essentially simi-

lar studies using HPV DNA detection assays with different degrees of reliability have obtained opposite conclusions [1, 2]. Variability in biologic stability over time will, unless corrected for, cause a systematic underestimation of relative risk in epidemiologic studies, known as regression dilution bias [13].

The present study has determined the extent of fluctuation of HPV antibody levels in serial samples from a population-based cohort of women. The fact that the antibody levels were mostly found to be stable suggests that seropositivity reflects lifetime exposure, which is in line with the fact that seroprevalences are strongly associated with lifetime number of sex partners (rather than with recent number of partners) [7] and with the lack of consistent age association of HPV seroprevalences [11].

In assessing generalizability of the present study, the fact that it was done among pregnant women should be considered, since pregnancy can reactivate certain viral infections. There is presently no evidence that pregnancy per se may affect HPV antibody levels, and both enrollment and follow-up samples were taken at the same trimester of pregnancy. If pregnancy per se would have an effect on HPV antibody levels, sampling of women during a time period when antibody levels are changing would have resulted in an increased variability of antibody levels between samples. The observed consistency of antibody levels argues against major changes in antibody levels during pregnancy. However, the possibility that antibody levels may be even more stable in follow-up studies of nonpregnant women cannot be excluded.

Previous studies of subjects with new HPV infection found that sometimes transient antibody peaks that return to seronegativity after clearance of viral DNA may be seen [10]. It is probable that very short-lived infections or virus exposures that do not result in infection might not induce a seroconversion to stable antibody levels, but when a sample of women not selected because of recent HPV infection is followed, mostly the women with sufficiently long-lasting infection to induce stable antibody levels are detected. HPV seropositivity is more common among women in whom HPV DNA has been detected on two occasions [9, 14]. Detection of HPV on two occasions some years apart also increases the probability that the HPV DNA test was not misclassified and increases the likelihood that the demonstration of viral DNA did indeed reflect an infection. Similarly, clearly detectable amounts of viral DNA (high virus load) are more likely to reflect a correctly classified infection.

In comparing the somewhat variable estimates of the sensitivity of HPV serology, it is noteworthy that the studies that found sensitivities of $\geqslant$50% [4, 5, 7–9] also were those in which the known attributes of HPV DNA detectability (such as dependence on recent partner change) and case-control differences between healthy women and women with cervical intraepithelial neoplasia were most clearly seen. As has been emphasized previously [1], in the absence of differential misclassification, high correlations do imply correct measurements. Thus, simultaneous use of measurements based on different principles, such as HPV serology, HPV DNA measurements, and behavioral questionnaires, can be used for validation of measurements against each other and a reliable identification of subjects with a history of HPV infection.

In summary, the present study has quantified the biologic variability of HPV antibody levels over time. The stability of HPV antibody levels over time has important implications for interpreting seroepidemiologic studies and for understanding HPV immunobiology.

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High Prevalence of Antibodies to Human Herpesvirus 8 in Relatives of Patients with Classic Kaposi's Sarcoma from Sardinia

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A survey for antibodies to a recombinant small viral capsid antigen (sVCA) of human herpesvirus type 8 (HHV-8) was conducted in Sardinia, one of the world's highest incidence areas for classic Kaposi's sarcoma (KS). Prevalence of antibodies to HHV-8 sVCA was greatest in patients with KS (95%), followed by family members (39%) and a Sardinian control population age- and sex-matched to the relatives (11%). Within families, prevalence of antibodies was about equal among spouses, children, and siblings of KS patients, a finding that raises the possibilities of intrafamilial person-to-person or vertical transmission. Antibodies were detected 2–3 times more frequently in males than in females. The data show that prevalence of antibodies to HHV-8 sVCA correlates with the distribution of classic KS in a high- incidence area. Clustering of seroprevalence within some families suggests the presence of familial risk factors for active HHV-8 infection.

Classic Kaposi's sarcoma (KS) is one of several distinct clinical and epidemiologic variants of the disease. Human immunodeficiency virus (HIV)–associated and endemic KS in Africa are thought to be transmitted through sexual contact with an infectious agent. A gammaherpes virus, variably known as Kaposi's sarcoma–associated herpesvirus or human herpesvirus type 8 (HHV-8), is the leading candidate etiologic agent [1–3]. The pathogenesis of classic KS is likely to be complex. Classic KS is characterized by a high incidence in certain geographic areas, for example, in specific regions of the Mediterranean countries of Greece, Italy, and Turkey, and by a low incidence in northern European countries, such as England and Sweden [4–6]. Geographic clustering could result from as-yet-undefined genetic or environmental factors, including predisposition to exposure to and reactivation of HHV-8. Moreover, classic KS is a disease of the elderly, with highest incidence in the sixth to eighth decades of life. This epidemiologic pattern is more consistent with reactivation of a latent virus or possibly de novo acquisition of an opportunistic infection as the result of waning immunocompetence.

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There is an association between HHV-8 and classic KS. Viral DNA was detected by polymerase chain reaction (PCR) in 50%–92% of blood mononuclear cells from patients with KS residing in Sicily, Greece, and Sardinia [7–9]. By contrast, HHV-8 genomes were found in the blood of zero to 11% of patients without KS from these areas. Patients with classic KS from northern Italy were universally seropositive to latent nuclear antigens found in HHV-8–infected cells, whereas only 4% of healthy blood donors from Milan in northern Italy were seropositive [10]. Similarly, 94% of patients with classic KS from Greece were seropositive to a recombinant capsid antigen; 12% of age- and sex-matched controls were seropositive [8]. Overall, these studies indicate a very high rate of active HHV-8 infection in patients with KS and suggest that active infection, as detected by the presence of serum antibodies or viral DNA in the blood, is present in a minority of healthy subjects.

One of the highest incidence areas of classic KS in the world is the northern region of the island Sardinia, located in the Mediterranean Sea west of the Italian mainland. In the period 1977–1991, the annual incidence of KS in Sardinia was 2.4 per 10^5 population in men and 0.8 per 10^5 population in women [4]. The 3-fold higher incidence of KS in men than in women is found in other regions with increased frequency of classic KS as well as in regions with sporadic KS [11]. The incidence in Sardinia is at least 10-fold higher than in northern Europe [11] and thus affords a unique opportunity to investigate the epidemiology of classic KS and to explore its relationship with HHV-8. We have focused on families of patients with KS since such studies might shed light on the mode of transmission and other risk factors. In a previous study, we found that HHV-8 DNA could be detected in peripheral blood mononuclear cells

(PBMC) of Sardinian KS patients, whereas their relatives were HHV-8–negative by this PCR assay [9]. We have now used sensitive and specific immunoblotting assays for antibodies to a recombinant small viral capsid component of HHV-8, as well as PCR, to determine the prevalence of infection in Sardinian patients with classic KS, in relatives, and in a control population from Sardinia that was age- and sex-matched to the relatives.

Materials and Methods

Study population. Serum was collected in 1996 from 20 patients with classic Kaposi's sarcoma, 17 males and 3 females, seen at the Department of Dermatology, University of Sassari. KS patients had a 5- to 10-year history of disease localized only to the skin. The majority lived in rural areas and worked as farmers or shepherds. Sera were obtained from 36 of their relatives, including spouses, children, and siblings, all of them living in Sardinia. The couples lived together for an average of 30 years, and the children lived with the family for ~25 years. Control subjects were 51 healthy persons and 40 patients with other diseases recruited in Sardinia for comparison with the relatives of KS patients. Target numbers for control subjects in various age and sex strata were calculated to match the characteristics of the relatives and to provide 2 controls for each subject. Target numbers for recruitment of control subjects were exceeded in several strata, particularly among elderly female controls. However, to avoid potential bias, all samples collected were included in the analysis.

Data collection and analysis. Clinical and demographic data were recorded on standardized forms that were linked to the serum samples by numeric code. KS patients, their relatives, and controls were compared using Student's t test for age, and χ^2 or Fisher's exact test for comparison of other characteristics. Odds ratios (ORs) were calculated with Cornfield 95% confidence intervals (CIs). Means are expressed ±SD. Statistical calculations were performed with Epi Info software (USD, Stone Mountain, GA).

Serologic assays. All serologic assays were performed and interpreted while the serum samples remained coded. The sera were tested by an immunoblot assay for antibodies to purified recombinant HHV-8 capsid antigen protein, designated sVCA, expressed in *Escherichia coli* [12]. Extensive studies have shown that human and rabbit antibodies to HHV-8 sVCA do not cross-react with its EBV homologue [12]. sVCA was cloned in pET30b (Novagen, Madison, WI), expressed in *E. coli* strain BL21 (DE3)-pLysS, and purified on a nickel column. Comparable fractions of a column containing *E. coli* extract transformed with the pET30 vector without an insert served as a negative control. Each lane of a 12% polyacrylamide gel was loaded with ~8 ng of antigen. The gels were blotted and incubated with sera that had been heated at 56°C for 30 min and diluted 1:100 in 5% skim milk. Immunoreactivity was detected by addition of 1 μCi of ^{125}I-labeled protein A. The blots were exposed to XAR film for 48 h. The presence of a band at 29 kDa in the sVCA lane and its absence in the control lane defined a positive reaction.

PCR assays for HHV-8 DNA. PBMC samples were assayed for HHV-8 sequences by a nested PCR technique [9]. Results of PCR analysis of samples from 10 patients and 11 relatives have been described previously, but the correlation with the serostatus of the persons was unknown [9]. An additional 33 samples from controls were studied.

Results

Seroprevalence of HHV-8 sVCA in Sardinian patients with classic KS. Twenty patients with classic KS were studied, including 17 males and 3 females. The mean age of the KS patients was 72.3 ± 10.6 years. Nineteen of the KS patients (95%) were seropositive. The 1 seronegative subject was a 68-year-old man who also had Castleman's disease. The presence of antibodies to KS was very strongly associated with clinical KS, whether comparing the KS patients with their relatives ($P < .001$) or with controls ($P < .001$).

Seroprevalence to HHV-8 sVCA among relatives of patients with classic KS. Seroreactivity to HHV-8 was significantly more prevalent among the relatives of KS patients (39% seropositive) than among the controls (11% seropositive; OR, 5.2; 95% CI, 1.8–14.8). A demographic comparison of the relatives of KS patients with the Sardinian controls (table 1) showed that the 2 groups were well matched in sex and age distribution. The higher seroprevalence among relatives was true for both men and women, although the association between seroreactivity and being the relative of a KS patient was stronger for women than for men. The relatives of KS patients included spouses, children, and siblings (table 2). Among relatives, the high rate of seroprevalence, which varied between 33% and 43%, was similar for spouses of an affected family member, for children, and for siblings (table 2). There was no significant difference between seroprevalence in spouses compared with other relatives. Furthermore, the association between seroreactivity and being a relative of a KS patient remained significant when only nonspousal relatives were considered ($P = .007$; OR, 4.63; 95% CI, 1.37–15.75).

Closer analysis of the 17 families of patients with KS provided evidence for clustering of the serologic response within families. Family members other than the index case were seropositive in 9 of the 17 families. In these 9 families, 14 (61%) of 23 relatives were seropositive. In 1 family, there were 4 seropositive family members, including 2 children and 2 siblings. In another family, there were 6 seronegative members, including 3 children and 3 siblings. Thus, the serologic response among family members did not seem to be randomly distributed. Additional evidence for clustering was the concordance between the serostatus of wives of affected patients and their children. In 2 families, both the mother and child were seropositive; in 5 families, the mother and her children were seronegative. Thus, among all 7 families in which sera from mothers and children were available, there was no example of a healthy seropositive mother with seronegative children or of a seronegative mother with seropositive children.

Table 1. Demographic characteristics and seroprevalence to HHV-8 recombinant small viral capsid antigen in Sardinia.

	Kaposi's sarcoma	Relatives	Controls	*P**
n	20	36	91	
Male	17 (85%)	9 (25%)	21 (23%)	NS
Female	3 (15%)	27 (75%)	70 (77%)	NS
Mean age (±SD)	72.3 (10.6)	53.9 (17.4)	57.9 (18.1)	NS
Male	72.9 (11.3)	55.3 (17.8)	67.3 (14.1)	NS
Female	73.7 (11.0)	53.4 (17.6)	55.1 (18.3)	NS
HHV-8 seropositive	73.2 (11.2)	60.2 (18.5)	60.2 (16.9)	NS
HHV-8 seropositive	19 (95%)	14 (39%)	10 (11%)	0.0003[†]
Male	16 (94%)	6 (67%)	5 (24%)	0.04
Female	3 (100%)	8 (30%)	4 (7%)	0.007

* Relatives vs. controls.
[†] Odds ratio = 5.15; 95% confidence interval = 1.82–14.81.

Effect of age and sex on seroprevalence. Since patients with KS were nearly universally seropositive, the effect of age and sex on seroprevalence could only be considered in the relatives and control population from Sardinia. Among relatives, the median age of those who were seropositive was 61 years, and that of those who were seronegative was 49.4 years; among the control subjects from Sardinia, the median age of those who were seropositive was 60.2 years, and that of those who were seronegative was 57.6 years. Although seropositive individuals tended to be slightly older than seronegative individuals, these differences were not statistically significant. In both the relatives and the controls, males were two to three times more frequently seropositive than females. This difference was not statistically significant among the relatives but was of borderline significance among the controls ($P = .05$).

PCR detection of HHV-8 in PBMC. As previously reported, a PCR signal for HHV-8 was detected in PBMC from patients with KS [9]. All 10 samples from KS patients were positive by PCR, including 1 from the patient who was seronegative. Among 11 relatives studied, none was positive by PCR, including 5 who were seropositive. None of the 33 control samples from Sardinia was positive by PCR; this group included samples from 6 persons who were seropositive. Thus, among relatives or control subjects, antibody screening was a more sensitive indicator of HHV-8 infection than was PCR-based detection of the viral genome in PBMC.

Table 2. Seroprevalence to HHV-8 recombinant small viral capsid antigen among relatives of patients with classic KS.

	Male	Female	Total	%
Spouse	1/1	5/13	6/14	43
Child	3/5	2/10	5/15	33
Sibling	2/3	1/4	3/7	43
Total	6/9	8/27	14/36	39

Discussion

This preliminary exploration indicates that the seroprevalence of HHV-8 infection in Sardinia mirrors the epidemiology of classic KS in several notable respects. The study demonstrates, using both serology and detection of viral DNA, the nearly invariable association of HHV-8 infection and classic KS in Sardinia. Relatives of patients with KS and the control population from Sardinia, a place of high incidence of classic KS, have a higher prevalence of antibodies to HHV-8 than does a population from central Italy, which is not a high incidence area for KS. We failed to detect any seropositive persons among 40 control subjects from Rome who were age- and sex-matched to the Sardinian relatives (data not shown). The prevalence of antibodies among relatives and healthy persons from Sardinia is 2- to 3-fold higher among males than among females, similar to the male-to-female ratio of 3:1 in the incidence of classic KS prior to the AIDS epidemic [11]. Among Sardinians, antibody to HHV-8, like KS, tends to be detected more frequently with increasing age, thus correlating with the higher incidence of classic KS in the elderly. However, a more detailed survey of age-specific prevalence of HHV-8 antibodies in the general population is needed before firm conclusions can be drawn.

A trend that emerges from this and other seroepidemiologic investigations is that there are three distinct patterns of HHV-8 seroprevalence among different patient groups within the same geographic region. Among patients with HIV-associated or classic KS, antibodies to HHV-8 are nearly invariant ([3, 12], this study). In a second, high-risk group, such as HIV-1–infected patients without KS, or in this study, relatives of patients with classic KS, the prevalence of antibodies is intermediate, from 20% to 40%. In the third group, the general population, seroprevalence is lower than in the high-risk group. However, the rate of seroprevalence in the general population varies dramatically within geographic regions; furthermore, this seroprevalence rate appears to correlate with the incidence

of the disease KS. In North America, northern Italy, and the United Kingdom, where KS is infrequent, seroprevalence is low, ranging from 1% to 5% [2, 8]. However, in Uganda, where KS is endemic, the prevalence of infection is 50%–60% in the general population [8, 10]. Places such as Greece and Sardinia, which are foci of classic KS, appear to have an intermediate HHV-8 seroprevalence of ~12% in the general population ([8], this study).

No previous studies have examined the serologic response of family members of patients with classic KS. Our study shows that there is a high seroprevalence of antibodies to HHV-8 among family members, >3-fold higher than in an age- and sex-matched Sardinian control group. The existence of a familial risk factor is suggested by clustering of seropositive persons within certain families. Since this higher rate extends to children and siblings as well as spouses, it is possible that there is vertical transmission or nonsexual person-to-person spread within families. The recent description of salivary shedding of HHV-8 provides a possible mode of intrafamilial transmission [14]. There may also be familial protective factors since lack of seroreactivity in the mother was associated with seronegativity in the children in 5 families. The nature of the factors that predispose to familial clustering of HHV-8 infection is not known; they may relate to viral strain, propensity for viral reactivation, or immune response to the virus. Some of these factors appear to be exportable: In places of low KS incidence, such as the United States and northern Europe, classic KS and posttransplant KS tend to occur in individuals of Mediterranean ancestry [15]. Familial clustering of HHV-8 infection may also account for the occasional occurrence of KS in families, usually in male siblings [16]. However, since HHV-8 infection is relatively common in the families of patients with classic KS in Sardinia, but familial classic KS itself is relatively rare, events in addition to HHV-8 infection must be needed for development of classic KS.

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GB Virus C Infection in Patients with Primary Antibody Deficiency

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Sera from 77 patients with common variable immunodeficiency (CVID) were tested for GB virus C (GBV-C) RNA, because they are prone to unexplained chronic hepatitis, and from 28 patients with X-linked agammaglobulinemia (XLA) who have a similar primary antibody deficiency but are not prone to hepatitis. Eight CVID and 8 XLA patients were positive; 6 positive CVID and 3 XLA patients had abnormal liver enzymes, explained in 3 by either hepatitis B or C virus infection. Most patients tested had antibodies to the E2 antigen of GBV-C, apparently passively acquired from their immunoglobulin therapy. The high prevalence of GBV-C viremia in CVID and XLA patients is probably explained by their long-term exposure to blood products. Our data indicate that GBV-C does not cause chronic hepatitis in immunocompromised XLA patients and is not the cause of chronic non-B or -C hepatitis in the majority of CVID patients.

There have been six reported clusters of hepatitis C virus (HCV) infection in patients with primary immunodeficiency since 1982. Five of these have been related to HCV contamination of batches of pooled immunoglobulin used in their therapy [1]. Recently, Quinti et al. in Italy [2] reported a high prevalence of HCV infection in patients with common variable immunodeficiency (CVID) that could not be linked to contaminated immunoglobulin. Although a subsequent study in the United Kingdom [3] found no evidence of unexplained HCV infection in similar patients, the Italian experience raises the question of whether HCV or related flaviviruses might be important in the pathogenesis of CVID, which is thought to be a polygenic disorder of immune regulation, possibly triggered by viral infection [4]. Other viruses that are spread by the parenteral route have recently been cloned and designated as GB virus C (GBV-C) [5] and hepatitis G virus (HGV) [6]; these are now known to be different isolates of the same virus. Both are known to be transmitted by blood transfusion, and one estimate puts the prevalence of viremia in the blood donor population at ~1% [7].

Hepatitis, sometimes leading to cirrhosis, occurs in about 10% of CVID patients in the United Kingdom [4]. Liver biopsy usually shows various degrees of periportal lymphocytic infiltration, sometimes associated with scattered granulomas. Some patients may have splenomegaly with multiple granulomas in the spleen and adjacent lymph nodes. We have screened sera from a large group of CVID patients for GBV-C infection, using a polymerase chain reaction (PCR) technique to identify viral RNA. Serum was also screened from patients with X-linked agammaglobulinemia (XLA), a disease that is caused by genetic mutations in the *btk* gene expressed in B lymphocytes and in which non-A, -B, and -C hepatitis is very rare [8].

Patients and Methods

Seventy-seven CVID patients (36 female) were studied; the mean age was 46 years (range, 19–83), and the mean time from diagnosis was 15 years. The diagnosis was based on the World Health Organization criteria [9]. Sera from 28 XLA patients, diagnosed by early onset of infection and very low or absent circulating B lymphocytes, were also screened; XLA was confirmed by genotyping in 18 patients. The mean age was 30 years (range, 2–45), and the mean time from diagnosis was 25 years. All patients were being followed up in the Medical Research Council Immunodeficiency Clinic, Royal Free Hospital, London. Sera were collected during the period from mid-1995 to the end of 1996, except from 7 patients (5 CVID, 2 XLA) who had died or left the UK; those samples had been stored for up to 20 years at −70°C. Serum was separated from blood within 6 h and stored at −20°C or −70°C until tested.

Reverse transcription (RT)-PCR for GBV-C RNA. Sera from 83 patients were tested as follows: GBV-C RNA was detected using an automated procedure (LCx GBV-C assay; Abbott Diagnostics, North Chicago) according to the manufacturer's instructions. Briefly, RT-PCR was used to generate amplified products from viral RNA in the clinical samples using a primer-probe set specific for the 5′UTR of the genome. An antisense oligonucleotide acts as the primer for cDNA synthesis and for the PCR reaction with a second (sense) oligonucleotide, the rTth polymerase catalyzing both reactions. A third oligonucleotide, which is complementary to one of the strands of the PCR product, acts as a probe, allowing capture of the product and subsequent detection via a hapten that is bound to the PCR primers (and products) and is recognized by an antibody conjugated to alkaline phosphatase. Sera from the remaining 22 patients were

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tested by either an in-house nested PCR based on the method described by Jarvis et al. [7] and/or that described by Linnen et al. [6]. The former method was compared with the LCx assay for 102 selected sera from patients with a variety of liver diseases, of which 51 were known to be positive in the LCx assay. There was >95% concurrence between these two assays.

GBV-C E2 antibody immunoassay. The primary assay was a direct binding assay in which an E2-coated polystyrene bead reacts with anti-E2 antibodies present in human samples, followed by the addition of horseradish peroxidase (HRPO)–goat anti-human IgG. To confirm the primary result, a sandwich assay was developed, in which an E2 coated bead reacts with anti-E2 antibodies in the sample, followed by addition of biotinylated E2 and then anti-biotin antibody conjugated to HRPO [10]. Sera were screened for hepatitis B virus (HBV) surface antigen by a routine method and for HCV RNA as previously described [3].

Results

Eight of 77 CVID and 8 of 28 XLA patients were positive for GBV-C RNA. Five positive sera were double-checked either by confirming positivity on a separate serum sample using a different PCR assay or by sequencing the PCR products to confirm their viral origin. Table 1 shows details of the CVID and XLA patients who were positive for GBV-C RNA. Three of the 8 positive XLA patients had consistently abnormal liver function tests, which were explained in 2 by either chronic HBV or HCV infection. In the 8 CVID patients who were positive, 6 had abnormal liver function tests, explained in 1 by chronic HCV infection.

GBV-C infection was associated with symptomatic chronic inflammatory bowel disease in 4 CVID patients and 2 XLA

Table 1. Features of GBV-C–positive patients.

Diagnosis, case no.	Current age, y	Age at diagnosis, y	Age when ALT/ALP first raised, y	Recent liver enzymes		Liver biopsy	Blood products	Other unusual features
				ALT	ALP			
Common variable immunodeficiency								
1	20	5	5	n	++	5 y: periportal lymphocytic inflammation, granulomas	a,c	Presented at 5 y with hepatosplenomegaly, lung granulomas, and lymphadenopathy; 20 y: splenectomy, granulomas in spleen
2	48	29	46	+	n	ND	a,b,c	Mild inflammatory bowel disease with normal jejunal villi
3	39	15	—	+	+	ND	a,c	Partial villous atrophy in jejunum, response to gluten-free diet; HCV-positive from contaminated IVIG
4	56	40	45	+	+	Granulomas in parenchyma (1987)	a,b,c	Previous mycoplasma arthritis
5	74	41	—	n	n	ND	a,c	
6	44	32	41	+	+	ND	a,c	Severe chronic inflammatory bowel disease, alopecia
7	36	35	—	n	n	ND	c	Steroid responsive thrombocytopenic purpura
8	52	16	12	+	+++	19 y: few lymphocytes in some portal tracts; 23 y: small foci of lymphocytes and polymorphs; 43 y: cirrhosis portal hypertension	a,b,c	12 y: hepatosplenomegaly; 19 y: splenectomy, lymphadenopathy, chronic inflammatory bowel disease; 50 y: chronic uveitis
X-linked agammaglobulinemia								
9	27	<1		n	n	—	a,b,c	Nil
10	35	1		n	n	—	a,b,c	Nil
11	38	2	31	+	+	Early cirrhosis (1997)	b,c	Crohn's-like bowel disease, HBsAg-positive
12	35	1	22	+	n	Mild hepatitis (1987), portal tract lymphocytic infiltration	a,b,c	HCV-positive from contaminated IVIG
13	22	5	—	n	n	—	a,b,c	Nil
14	26	<1	—	n	n	—	a,c	Chronic mycoplasma joint infections
15	34	8	—	n	n	—	a,c	Nil
16	32	9	28	n	+++	ND	a,b,c	Chronic inflammatory bowel disease with partial jejunal villous atrophy

NOTE. ALT, alanine aminotransferase (+ = 40–100); ALP, alkaline phosphatase (+ = 130–200, ++ = >200, +++ = >300 IU/L); y, years; n, normal; a, intramuscular immunoglobulin; b, fresh frozen plasma; c, intravenous immune globulin (IVIG); ND, not done; HCV, hepatitis C virus; HBsAg, hepatitis B surface antigen.

patients, compared with 9 with bowel disease in the remaining 69 GBV-C–negative CVID patients and none of the remaining 26 XLA patients. Overall, 38% of GBV-C–positive patients had symptomatic bowel disease, compared with 8% who were GBV-C–negative (table 2). There were 26 CVID patients with signs of chronic hepatitis who were GBV-C–negative. In 3 of those 26, this was explained by HCV infection, and 2 of those 3 developed cirrhosis. However, there were 3 HBV–, HCV–, and GBV-C–negative patients who died from cirrhosis.

Most of the patients had been treated since diagnosis of their immunodeficiency with regular immunoglobulin therapy for many years at 2-week intervals. All but 1 of the patients who were GBV-C–positive had received substantial amounts of intravenous immune globulin (IVIG) over many years, and many had received fresh frozen plasma (FFP) therapy before IVIG was introduced into the United Kingdom in 1983. In general, the XLA patients had had immunoglobulin therapy, including FFP, for longer than the CVID patients (mean, 25 vs. 15 years).

Fifty-eight patients (10 XLA, 48 CVID) were tested for antibodies to E2, and only 7 were negative; 4 of the negative patients had never received immunoglobulin therapy, 2 were on low-dose immunoglobulin, and only 1 was on standard IVIG (200 mg/kg every 2 weeks), in contrast to the remaining 51 who were on standard IVIG therapy. Nine patients were both viremic for GBV-C and seropositive for E2; 1 patient who had not received immunoglobulin treatment was viremic and seronegative.

Six separate batches of IVIG (3 of Sandoglobulin [Novartis, Switzerland] and 3 of Alphaglobin [Alpha Therapeutic, Barcelona, Spain]) were tested for antibodies to E2 after 2-fold dilutions from 1:5 to 1:100 were made of the 5% or 6% infusion solutions. All were positive at the 1:5 dilution, with variation in positivity to the higher dilutions between batches, indicating different levels of antibody in these batches. Six batches each of Alphaglobin and Sandoglobulin were tested for GBV-C RNA with the LCx PCR assay, and all were negative.

Discussion

There is clearly a raised prevalence of GBV-C infection in patients with primary antibody deficiency compared with the general population, with 10% of CVID and 29% of XLA patients in our series being positive. This may not be surprising in view of the substantial amounts of blood products that these patients have received over many years. Furthermore, it is likely that many of those patients who were diagnosed before 1980 acquired the infection from regular FFP therapy. This may explain the particularly high prevalence in XLA patients, since many of them received plasma in the 1970s before IVIG was available. It is also possible that some patients acquired infection from contaminated IVIG therapy, as a recent report has shown that some batches from one particular product were positive for GBV-C when tested by PCR for viral RNA [7]; we found no evidence of this in preparations currently used in our clinic. The high prevalence of GBV-C contrasts with our previous finding that only 3 of 44 patients, most of whom were included in this study, were negative for HCV RNA [3].

The presence of 23 CVID patients in our series with chronic hepatitis that could not be explained by HBV, HCV, or GBV-C infection suggests that these patients are prone to another hepatitis virus. Unlike XLA patients, CVID patients have a functional defect in CD4 T lymphocyte antigenic responses and may be prone to certain viruses [4]. However, many CVID patients also display features of immune dysregulation, such as their circulating lymphocytes having the capacity to produce high levels of interferon-γ and TNF-α [11]. This, together with the finding of granulomas in the livers of some of these patients suggests an inappropriate inflammatory response to chronic infection, GBV-C being one possible candidate.

The finding that the majority of patients were negative for GBV-C, despite many years of IVIG treatment, shows that if infection occurs from contaminated IVIG, it is rare. If contamination of IVIG has occurred over the past 15 years, the small number of infected patients could be explained by the virus being present as an antibody-virus complex that may neutralize infectivity. Experience with HCV-contaminated IVIG suggests that patients were partially protected from infection when the immunoglobulin contained specific antibody [12]. This is supported by our finding that all six batches of IVIG tested contained some antibody to E2; it is likely that all current commercially available batches of IVIG will contain antibodies to E2, as the starting plasma pools for individual batches usually con-

Table 2. Association of gut and liver disease with GBV-C viremia.

		GBV-C–positive			GBV-C–negative		
	Total tested	Total no. (%)	No. with raised liver enzymes	No. with IBD	Total no.	No. with raised liver enzymes	No. with IBD
CVID	77	8 (10)	6 (1 HCV)	4	69	26 (3 HCV)	9
XLA	28	8 (29)	3 (1 HCV) (1 HBV)	2	20	4 (3 HCV)	0

NOTE. IBD, inflammatory bowel disease; CVID, common variable immunodeficiency; XLA, X-linked agammaglobulinemia; HCV, hepatitis C virus; HBV, hepatitis B virus.

tain 10,000–20,000 units from different donors, and a current estimate is that ~9% of the population are seropositive [10, 13]. This explains why nearly all our patients on IVIG are seropositive to E2.

About 10% of CVID patients suffer from symptomatic inflammation of the bowel, but this is rare in XLA [14]. Our data suggest an association between chronic GBV-C infection and bowel inflammation, with the virus possibly targeting cells within the gastrointestinal tract. The apparent genetic predisposition to chronic inflammation in CVID might explain this association. It is still not known which cell type is targeted by GBV-C, and cells of the lymphoid system have been suggested as a candidate [15]. However, we can exclude the B lymphocyte as an important reservoir because of our finding of GBV-C infection in XLA patients without B cells.

Although the high prevalence of GBV-C infection in immunocompromised XLA patients is of some concern, particularly if this is linked to contaminated immunoglobulin therapy, we have evidence against this causing significant chronic hepatic disease. In CVID, GBV-C infection is not the cause of the majority of non-B or -C hepatitis in these patients, and since some viremic patients have no evidence of hepatitis, it is doubtful whether this agent can cause liver disease in these patients.

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GB Virus C/Hepatitis G Virus Infection: A Favorable Prognostic Factor in Human Immunodeficiency Virus–Infected Patients?

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To investigate a possible influence of GB virus C (GBV-C) in immunocompromised patients, the prevalences of GBV-C RNA and anti–E2 antibody in 197 human immunodeficiency virus (HIV)–infected patients and in 120 control blood donors were studied. GBV-C RNA was detected in 33 of 197 HIV-infected patients (16.8%) compared with 1 in 120 blood donors (0.8%) ($P < .001$). Previous exposure to GBV-C (anti–E2 antibody–positive) was shown in 56.8% of HIV patients and in 9% of blood donors. GBV-C viremia was not associated with hepatitis. Despite approximately equal duration of HIV infection in all subgroups, the CD4 cell counts were significantly higher in GBV-C–viremic patients (344 cells/μL) compared with exposed (259 cells/μL) and unexposed (170 cells/μL) patients ($P = .017$ and $P < .001$). Furthermore, Kaplan-Meier analysis demonstrated significantly better cumulative survival in GBV-C RNA–positive HIV-infected patients, suggesting that GBV-C might be a favorable prognostic factor in HIV disease.

Coinfection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is a common complication in human immunodeficiency virus (HIV) infection, having implications on the outcome of affected patients [1, 2]. Recently, 2 new virus isolates related to the flaviviridae family, GB virus C (GBV-C) and hepatitis G virus (HGV), were identified in human sera, some from patients with cryptogenic or posttransfusion hepatitis [3, 4]. GBV-C and HGV are closely related isolates of the same virus, with >95% sequence homology [5]. Although the new virus was detected in patients with various forms of liver disease, its wide distribution among healthy persons made its clinical significance as a hepatitis agent uncertain [4, 6–8]. However, no data are available, to our knowledge, concerning the implication of GBV-C in HIV infection. Therefore, we retrospectively studied the seroprevalences of GBV-C RNA and anti–E2 antibodies in HIV-infected patients and correlated the results with the clinical follow-up data of the patients. The anti–E2 antibody is directed against the envelope 2 protein of GBV-C and was recently demonstrated to indicate exposure to the virus. Its appearance is mostly associated with viral clearance (GBV-C RNA–negative by reverse transcriptase–polymerase chain reaction [RT-PCR]) [9, 10].

Patients and Methods

We enrolled 197 HIV-infected patients who regularly attended our outpatient clinic between January 1993 and December 1994. The stage of disease was classified according to the revised Centers for Disease Control and Prevention (CDC) staging system with respect to the European modification of this classification. One hundred twenty randomly selected blood donors from our blood bank served as controls.

Serologic studies for the detection of HIV, HBV, and HCV. HIV antibodies were detected using an ELISA (Abbott Laboratories, Abbott Park, IL). Positive ELISA results were confirmed by Western blot. All participants were tested for anti–HCV antibodies (Abbott 2nd generation test), and positive sera were also tested for HCV-RNA by nested PCR of the 5′ untranslated region. Virus markers for HBV infection were evidence of hepatitis B surface antigen and hepatitis B core antibodies (Abbott Laboratories).

CD4 lymphocytes were measured by fluorescence-activated cell analysis (FACScan; Becton Dickinson, Heidelberg, Germany).

Detection of GBV-C RNA. RNA extracted from the equivalent of 5 μL of serum was amplified in a single-tube RT-PCR as previously described [11]. Briefly, cDNA synthesis was performed at 60°C for 30 min using rTth polymerase (Roche Molecular Systems, Branchburg, NJ) and a gene-specific antisense primer, followed by 35 cycles of amplification in a two-temperature PCR protocol (94°C for 40 s, 63°C for 60 s). PCR products were hybridized at 15°C for 10 min to an internal GBV-C–specific oligonucleotide probe. Sequences of sense and antisense primers and the hybridization probe were deduced from the 5′ untranslated region of GBV-C [11]. PCR amplicon-probe complexes were specifically detected by a microparticle capture EIA (Abbott Laboratories). Two dilutions of a GBV-C–positive serum served as a positive control, and a GBV-C–negative serum served as a negative control. The specificity of the method was demonstrated by a reference PCR and dot-blot hybridization using different primers and a different hybridization probe [11]. The detection limit of the assay was 100 molecules of an in vitro transcript per reaction.

EIA for the detection of anti–E2 antibodies. All sera were investigated by an EIA (Abbott Laboratories) for the presence of the anti–E2 antibody, which is directed against the E2 part of the presumed envelope protein of GBV-C [10].

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Statistical analysis. Categorical variables were analyzed by the χ^2 test. Group means were compared by the Student's *t* test or the Mann-Whitney *U* test, if appropriate. Cumulative patient survival was assessed by Kaplan-Meier analysis. Equality of survival distribution was calculated by log-rank test. $P < .05$ was considered statistically significant. The Cox proportional-hazard regression model was performed to calculate relative risks in a multivariable model including categories for sex, age (<36 years or ⩾36 years), detection of GBV-C RNA (positive or negative), anti–E2 antibody status (positive or negative), HBV status (hepatitis B surface antigen–positive or –negative), and HCV status (anti–HCV antibody–positive or –negative) [12].

Results

Prevalences of hepatitis B and C and GBV-C. Among 197 HIV-infected patients, 20 (10.2%) were hepatitis B carriers (HBsAg-positive), and 51 (25.9%) were HCV antibody–positive. HCV RNA was detected in 39 of these 51 HCV antibody–positive patients. Three patients (1.5%) were coinfected with HBV and HCV. GBV-C RNA was detectable in 33 of 197 HIV-infected patients (16.8%) and in 1 of 120 reference group blood donors (0.8%; $P < .001$). Analysis of all sera for the presence of the anti–E2 antibody (by EIA) revealed a seroprevalence of 56.8% (112/197). In comparison, only 11 of 120 (9%) blood donors were positive for the anti–E2 antibody ($P < .001$). None of the antibody-positive patients had detectable GBV-C RNA levels. Only 52 of 197 HIV patients (26.4%) had no markers of GBV-C infection. With respect to GBV-C status, we differentiated viremic (GBV-C RNA–positive), exposed (anti–E2 antibody–positive/GBV-C RNA–negative), and unexposed (anti–E2 antibody–negative/GBV-C RNA–negative) HIV-infected patients.

Transmission risk factors for an infection with GBV-C were deduced from the viral prevalence in the HIV risk groups. Intravenous drug use (51.5%) and homosexuality (39.4%) were the main risk factors in our patients (table 1).

Implication of GBV-C viremia and anti–E2 antibody prevalence. No significant differences were found in means of aminotransferases (aspartate aminotransferase, alanine aminotransferase), albumin, or cholinesterase levels between virus-positive, exposed, and unexposed patients. On average, viremic patients were younger than exposed patients (33.9 ± 8.8 years vs. 40 ± 10.3 years; $P = .006$), but they did not significantly differ from unexposed patients. However, GBV-C RNA–positive patients had significantly higher CD4 cell counts than patients without any exposure to GBV-C (344 cells/μL ± 189 vs. 170 ± 211; $P < .001$) or to anti–E2 antibody–positive patients who had already cleared the virus (259 cells/μL ± 270; $P = .017$). In analyzing subgroups in those GBV-C RNA–positive patients without HBV or HCV coinfection, higher CD4 cell counts were also evident compared with GBV-C RNA–negative patients of the same subpopulation.

Of 197 HIV-infected patients, 38 (19.3%) were classified as CDC stage I, 81 (41.1%) as CDC stage II, and 78 (39.6%) as CDC stage III. CDC stage analysis of the 33 GBV-C RNA–positive patients revealed the following distribution: 13 CDC stage I (39.4%), 19 CDC stage II (57.6%), and 1 CDC stage III (3%). In comparison, 40.1% of the exposed patients and 61.6% of the patients without exposure to GBV-C were diagnosed as CDC stage III. Significantly fewer patients had progressed to CDC stage III in the GBV-C RNA–positive group ($P < .001$).

Total RNA of peripheral blood mononuclear cells (PBMC) from 3 viremic patients in the CDC stage I subgroup was investigated by strand-specific reverse transcription followed by RNase H digestion and nested PCR of the 5′ untranslated region. Only positive- (not negative-) strand GBV-C RNA was detected in all 3 samples.

Survival analysis. For this analysis, we calculated the period from the first positive HIV test, available for 179 of 197 patients, to a cutoff date or death, because the exact date of HIV seroconversion was not known. Follow-up data for 28 GBV-C RNA–positive patients, 104 anti–E2 antibody–positive patients, and 47 patients without exposure to GBV-C revealed that HIV infection was of approximately equal duration in all groups before serum for the GBV-C test was taken (1530 ± 1403 days, 1490 ± 1224 days, and 1302 ± 1132 days, respectively; difference not significant). Mean survival time in the GBV-C RNA–positive group of HIV patients was 4590 ±

Table 1. Prevalence of GBV-C RNA and anti–E2 antibody in HIV-infected patients in relation to risk factors for virus transmission.

	All HIV-infected patients		GBV-C RNA–positive patients		Anti–E2 antibody–positive patients	
Risk factor	Frequency	%	Frequency	%	Frequency	%
Homosexual promiscuity	104	52.8	13	39.4	59	52.7
Intravenous drug use	60	30.5	17	51.5	31	27.7
Heterosexual promiscuity	26	13.2	1	3.0	18	16.1
Hemophilia	7	3.5	2	6.1	4	3.5
Total	197	100	33	100	112	100

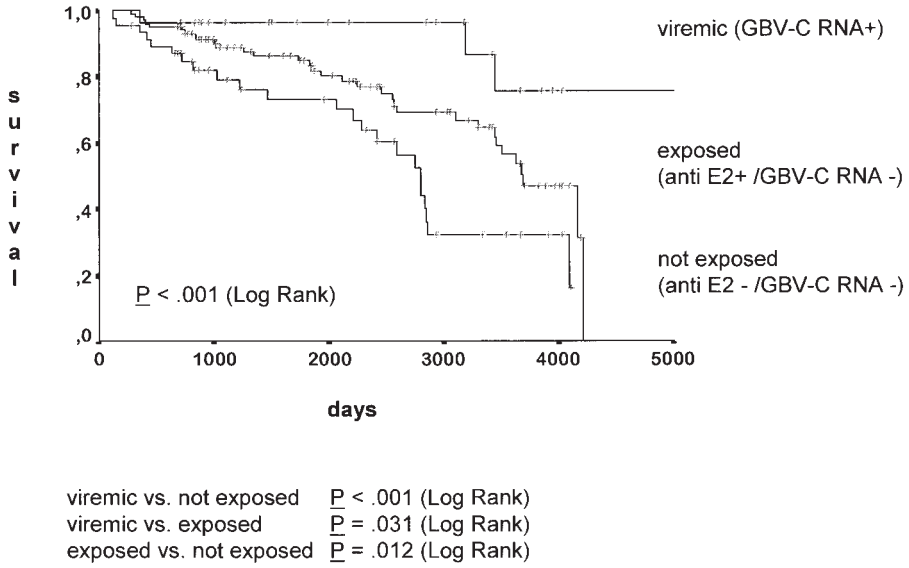

Figure 1. Kaplan-Meier survival analysis of HIV-infected patients relative to GBV-C status: GBV-C viremia (GBV-C RNA–positive), exposure to virus (anti–E2 antibody–positive, GBV-C RNA–negative), and no evidence of GBV-C infection (anti–E2 antibody–negative, GBV-C RNA–negative). Nos. of surviving patients were as follows: 25 (89%) of 28 in the GBV-C–viremic patients, 71 (68%) of 104 in exposed patients, and 24 (51%) of 47 in unexposed patients. Survival time was calculated from date of first positive HIV test (available for 179 patients). HIV infection was of equal duration in all groups before serum for GBV-C serology was taken. Survival difference is statistically significant; $P < .001$ (log-rank test).

279 days (95% confidence interval [CI], 4043–5137) compared with 3239 ± 143 days (95% CI, 2959–3519) in the anti–E2 antibody–positive group ($P = .031$, log-rank test) and with 2591 ± 217 days (95% CI, 2165–3017) in the unexposed patients ($P < .001$, log-rank test). The Kaplan-Meier survival analysis is illustrated in figure 1.

In the absence of detailed information about acquisition of GBV-C infection, we additionally analyzed the mean survival beginning from the date when serum was taken for analysis of the GBV-C status. For these calculations, all 197 HIV-positive patients could be considered. Twenty-nine (87.9%) of 33 GBV-C RNA–positive patients had survived beyond the cutoff date compared with 77 (68.8%) of 112 anti–E2 antibody–positive patients and with 29 (55.8%) of 52 patients without exposure to GBV-C. Mean survival of the GBV-C RNA–positive patients was 1052 ± 42 days (95% CI, 970–1134) versus 930 ± 42 days (95% CI, 847–1013) in the antibody-positive group ($P = .041$, log-rank test) and versus 777 ± 67 days (95% CI, 646–909) in the unexposed group of patients ($P = .002$, log-rank test).

A Cox proportional-hazard regression model indicated that GBV-C RNA (hazard ratio, 0.22; $P = .007$) is an independent factor associated with a relative benefit on survival more distinctly than the presence of the anti–E2 antibody (hazard ratio, 0.52; $P = .017$). Age of >36 years as well as HBV infection were associated with relative risks of death (hazard ratio, 2.68; $P = .006$ and hazard ratio, 2.26; $P = .029$, respectively). Gender and HCV antibody status had no prognostic value for the patients studied.

Discussion

We found a significantly increased GBV-C RNA prevalence in the HIV-infected patients compared with the control group blood donors (16.8% vs. 0.8%). The prevalence in our control group corresponds to that of volunteer blood donors reported by others [4, 7, 8].

Major transmission risk factors for infection with GBV-C were intravenous drug use and homosexuality. In view of the report by Persico et al. [13], who demonstrated the presence of GBV-C RNA in cell-free seminal plasma, the high prevalence of GBV-C RNA in homosexuals in our study is understandable.

In some studies, HBV and HCV infection were demonstrated to worsen the clinical course and the outcome of HIV-infected patients [1, 2]. We found no hepatitis (as measured by increased transaminases) associated with GBV-C infection in the immunocompromised patients. Mean levels of transaminases, slightly above the upper normal range, did not differ between patients with CDC stage I, II, or III of HIV infection, indicating the same degree of liver damage in all three CDC stages.

Of interest, GBV-C–viremic patients had distinctly higher CD4 cell counts and progressed less frequently to CDC stage III compared with exposed and unexposed patients. However, HIV infections were of approximately equal duration in all subgroups up to the time when samples were taken for GBV-C serology. Kaplan-Meier analysis indicated significantly better cumulative survival after the first positive HIV test in the GBV-C–viremic patients (figure 1). Although GBV-C RNA–positive patients were significantly younger than antibody-positive patients, they were about the same age as the unexposed patients. A multivariable Cox regression analysis confirmed that GBV-C viremia was associated with a relative benefit on survival in the HIV-infected patients independent of other variables such as age. However, the number of GBV-C RNA–positive HIV-infected patients in our study ($n = 33$) is relatively small, and these results need confirmation in larger studies. Analyses of sequential samples might help to elucidate whether GBV-C and HIV are simultaneously acquired by

shared transmission risk factors or whether one of the viruses is contracted first.

An important question for the future is whether CD4 cells are a replication site of GBV-C because decreased CD4 cell counts could explain the significantly fewer GBV-C–positive patients in CDC stage III of HIV infection. We attempted to detect viral negative-strand RNA as replication intermediate in lymphocytes of GBV-C–positive patients. However, in agreement with data from others [14], we could detect genomic but not antigenomic viral RNA in 3 PBMC samples of viremic HIV-infected patients from the CDC stage I subgroup. Further studies are necessary to investigate a possible tropism of GBV-C in PBMC subsets in detail.

Recently, relative resistance of CD4 lymphocytes to HIV infection was described in persons with high-risk sexual exposure [15]. The high prevalence of GBV-C in the homosexual risk group of our study together with the frequent detection of GBV-C in seminal plasma [13] are interesting observations in this context.

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Cross-Clade Inhibition of Human Immunodeficiency Virus Type 1 Primary Isolates by Monoclonal Anti-CD4

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A murine monoclonal antibody (MAb) with human CD4 specificity was tested for the ability to inhibit primary human immunodeficiency virus type 1 (HIV-1) isolates clades A through E. Human peripheral blood mononuclear cells (PBMC) were used as target cells for infectivity. The HIV-1 primary isolates were examined for the capacity to infect PBMC targets in the presence or absence of the anti-CD4 MAb, designated P1. P1 broadly inhibited clade A, C, D, and E isolates, based on a reduction of HIV-1 p24 antigen concentrations compared with untreated controls. Little to no virus-inhibiting activity was observed with a primary HIV-1 clade B isolate, designated BZ167. Additionally, a second primary clade B isolate was efficiently inhibited from infecting PBMC targets by P1. The data indicate that P1 exhibits group-specific inhibiting activity against non–clade B primary HIV-1 isolates in vitro.

Primary isolates from all clades of human immunodeficiency virus type 1 (HIV-1) are relatively resistant to inhibition by serum antibodies, irrespective of the clade infecting the serum donor [1]. In addition, vaccine candidates tested to date have failed to induce antibodies able to inhibit primary HIV-1 isolates across all identified clades [2–4], and inhibition by HIV-1–positive sera both within and across clades tends to be highly variable [5, 6].

HIV infection is initiated by contact between the virus envelope glycoprotein, gp120, and the T cell receptor, CD4. Since the initial binding step of all HIV isolates occurs through this gp120/CD4 interaction, antibodies specific for CD4 might be effective against multiple HIV-1 isolates. It has been shown that some anti-CD4 monoclonal antibodies (MAbs) bind to CD4 via a site close to that binding gp120 and prevent subsequent binding of HIV-1 laboratory isolates to CD4 [7] (reviewed in [8]). In addition, it has been shown that HIV-1–infected patients may be treated with anti-CD4 MAb without any apparent deleterious effects [9]. Chimeric murine/human anti-CD4 has also been infused into patients in milligram quantities, for weeks at a time, with no significant depletion of CD4-positive cells and no alteration of the phenotype of peripheral blood lymphocytes [10]. In this set of experiments, we used a murine anti-CD4 MAb, designated P1, to test neutralization of various HIV-1 primary isolates across clades.

Materials and Methods

Virus stocks. HIV-1 primary isolates were obtained from Quality Biologicals (Gaithersburg, MD), the Aaron Diamond AIDS Research Center (New York), and the AIDS Research and Reference Reagent Program (Rockville, MD). Stocks were expanded in fresh human peripheral blood mononuclear cell (PBMC) blasts by weekly coculture. Briefly, 2×10^6 blasts were washed in saline and pelleted. Cell pellets were infected with 200 μL of virus stock for 2 h at 37°C with shaking every 15 min. After 2 h, the virus inoculum was washed away with saline, and the infected cells were resuspended in 2 mL of RPMI/interleukin-2 medium and plated into an individual well of a 24-well tissue culture plate. On day 4, 1 mL of the culture was removed and replaced with 1 mL of fresh medium. On day 7, 1 mL of culture was removed and replaced with 1 mL of 2×10^6 fresh blasts. The following HIV-1 isolates were used in neutralization assays: UG029 (Uganda) subtype A, BZ167 (Brazil) subtype B, A284 (Argentina) subtype B, IN905 (India) subtype C, UG001 (Uganda) subtype D, and KH005 (Thailand) subtype E. Each of the isolates induces syncytia in PBMC, and the stock titers ranged from 10^3 to 10^5 $TCID_{50}$/mL.

Antigen-capture ELISA. To quantify p24 levels in our experiments, we used a modification of an HIV-1 p24 antigen assay (Coulter, Miami). Briefly, individual wells of 96-well microtiter plates were coated with 50 μL of MAb anti-p24 (10 μg/mL) diluted in borate-buffered saline (BBS) for 1 h at 37°C. The unbound sites were blocked by addition of 200 μL of 5% goat serum diluted in BBS (GSBBS) for 30 min at 37°C. The wells were then washed five times with BBS containing 0.05% Tween 20 (BBST). Samples (50 μL) were loaded and incubated for 1 h at 37°C and washed five times with BBST. The anti-p24 biotin reagent from the kit was then diluted 1:4 in GSBBS, and 50-μL volumes were added to the wells for 1 h at 37°C. Unbound material was washed away with five washes with BBST. Finally, 50 μL of a predetermined dilution of streptavidin–horseradish peroxidase diluted in GSBBS was added for 30 min at 37°C. Unbound streptavidin–horseradish peroxidase was washed away with BBST, and the assay was developed by addition of 50 μL of tetramethylbenzidine. The development was stopped after 30 min by addition of 20 μL of 1 *M* HCl, and the assay was read at 450 nm in a microplate reader. Each point was assayed in duplicate, and the linear range of this modified HIV-1 p24 assay, based on optical density at 450 nm, was 0.12–

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1.40. This corresponded with an HIV-1 p24 concentration of 10–600 ng/mL as determined from the linear portion of the standard curve (data not shown).

$TCID_{50}$ determination. HIV-1 stocks were serially diluted (1:10) in saline, and 2×10^6 PBMC blasts were infected for 2 h at 37°C. The virus inoculum was washed away with saline, and the cells were resuspended in 1 mL of medium. The cells were then aliquoted into 10 wells of a 96-well tissue culture plate (100 μL/well). On day 4, 100 μL of fresh medium was added, and on day 7, the wells were tested for p24. The $TCID_{50}$/mL was determined by the Reed-Muench method.

Cell viability assay. To evaluate the viability of our PBMC preparations, we used MTT as an indicator of metabolic activity. Briefly, samples of cells from our neutralization experiments were harvested, and 0.1 vol of MTT (5 mg/mL) in RPMI was added for 4 h at 37°C. Converted dye was then solubilized with an equal volume of 1 *M* HCl and vigorous pipetting. The assay was measured spectrophotometrically on an ELISA plate reader at 570 nm.

MAb P1. P1 is a murine MAb generated by the fusion of SP2/0 myeloma cells with BALB/c immune splenocytes. P1 is an IgG1/k, was produced as an ascitic tumor, and was subsequently purified on protein A–sepharose by standard procedures. P1 has been shown to be identical to Leu-3a in epitope specificity. In addition, P1 varies by only 2 conserved amino acids in the heavy-chain variable region and is identical in light-chain variable region amino sequence to Leu-3a [11].

Neutralization assay. Fresh 3 day–blasted human PBMC, 2×10^6/experiment, were harvested and washed two times in saline. Cells were pretreated or not with the anti-CD4 MAb, P1, or isotype-matched control MAb and pelleted before infection for 2 h at 37°C with 200 μL of HIV stock in medium. Infected cells were shaken every 15 min during the 2-h infection, after which the PBMC were pelleted, and unadsorbed virus was washed away with saline. Finally, the infected cells were resuspended in 2 mL of RPMI/interleukin-2 medium and added to individual wells of 24-well tissue culture plates. Cells were fed on days 4, 7, and 10 by removing 1 mL of culture and replacing it with 1 mL of fresh medium. The level of infectivity was determined on the basis of optical density values from the HIV-1 p24 ELISA.

Results

Kinetics of viral replication. Before examining the ability of P1 to inhibit the various primary isolate clades, it was necessary to determine the kinetics of HIV-1 replication in human PBMC. We infected phytohemagglutinin-blasted PBMC as described above. Inocula of 1, 10, and 100 $TCID_{50}$/mL were tested. Samples were taken at days 4, 7, and 10 and assayed by p24 antigen capture. In figure 1, the kinetics of HIV-1 (UG001) replication are depicted. On the basis of HIV-1 p24 levels, at 100 $TCID_{50}$/mL inoculum, virus titers are maximum and constant from days 4, 7, and 10. At 10 $TCID_{50}$/mL, the virus titers steadily increase from day 4 to day 10. At 1 $TCID_{50}$/mL, the titers are not sufficient even at day 10. For evaluating the inhibitory activity of the anti-CD4, a 100 $TCID_{50}$/mL inoculum was selected for further studies. We performed similar studies with the other HIV-1 isolates (data not shown) and also found 100 $TCID_{50}$/mL to be optimal.

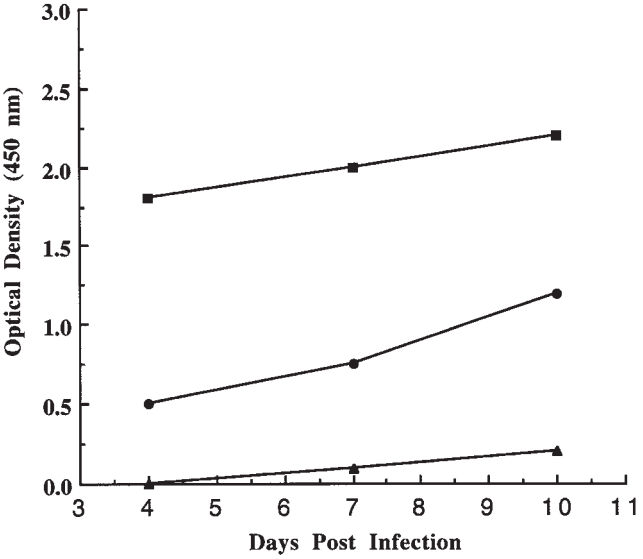

Figure 1. Kinetics of HIV-1 primary isolate replication in human PBMC. Antigen-capture ELISA was used to estimate HIV-1 p24 levels in infected PBMC; 100 (■), 10 (●), and 1 (▲) $TCID_{50}$/mL inocula were tested to examine kinetics of viral replication over 10-day period and determine dose for neutralization experiments.

Effect of P1 on PBMC viability. Before testing for inhibition, it was necessary to test whether P1 itself had any toxic effect on the PBMC blasts. To evaluate toxicity, we used the MTT cell viability assay. No significant decrease in viability was observed in the presence of P1 at 10 and 100 μg/mL (data not shown). The results indicate that P1 is not toxic to the PBMC blasts that will serve as targets for subsequent infectivity assays.

Inhibition of HIV-1 primary isolates by MAb anti-CD4. Levels of HIV-1 p24 in cultures of human PBMC infected with representative primary isolates in the presence or absence of anti-CD4 MAb P1 are shown in table 1. On days 4, 7, and 10, the clade A isolate was inhibited with 10 μg/mL by 91%, 7%, and 0, and with 100 μg/mL by 91%, 100%, and 98%, respectively. Clade B (BZ167) was inhibited with 10 μg/mL by 54%, 0, and 0, and with 100 μg/mL by 91%, 15%, and 12%, respectively. The clade C isolate was inhibited with 10 μg/mL by 76%, 97%, and 91%, and with 100 μg/mL by 84%, 95%, and 98%. Similarly, 10 μg/mL decreased clade D by 100%, 93%, and 90%, and 100 μg/mL by 100%, 100%, and 100%, respectively. The clade E isolate was inhibited by 91%, 99%, and 98% and by 90%, 98%, and 98% with 10 μg/mL and 100 μg/mL, respectively. In replicate cultures that contained the same concentrations of a control MAb specific for hepatitis B surface antigen, inhibition of HIV-1 infectivity was $<$15%. To address the issue regarding the lack of efficient inhibitory activity against the clade B isolate, we examined a second clade B isolate, along with other primary isolates

Table 1. Levels of p24 in cultures of human peripheral blood mononuclear cells infected with HIV-1 primary isolates in presence of monoclonal anti-CD4.

Days after infection	Anti-CD4 (μg/mL)	Clade									
		A		B		C		D		E	
		OD	% inhibition	OD	% inhibition	OD	% inhibition	OD	% inhibition	OD	% inhibition
4	0	0.011	0	0.751	0	0.983	0	0.458	0	1.143	0
	10	0.001	91	0.344	54	0.233	76	0.001	100	0.106	91
	100	0.001	91	0.063	92	0.158	84	0.001	100	0.111	90
7	0	0.332	0	0.913	0	1.149	0	0.727	0	0.980	0
	10	0.308	7	0.907	1	0.035	97	0.053	93	0.014	98
	100	0.001	100	0.772	15	0.057	95	0.001	100	0.023	98
10	0	0.806	0	0.714	0	1.127	0	1.070	0	0.874	0
	10	0.860	0	0.694	0	0.105	91	0.108	90	0.018	98
	100	0.014	98	0.627	12	0.027	98	0.001	100	0.014	98

NOTE. Levels of p24 were determined by antigen-capture ELISA. Optical densities (ODs) were determined at 450 nm (means of duplicate determinations). % inhibition is in comparison to control infected values.

representing the other clades. P1 efficiently inhibited the second clade B isolate (A284), similar to the levels of inhibition observed with the representative clade A, C, D, and E isolates (data not shown).

Discussion

In this study, we examined the inhibition potential of an anti-CD4 MAb, P1, on HIV-1 primary isolates. We first determined, in vitro, that P1 did not affect the viability of the PBMC preparations that were used as target cells in the inhibition assay. The inhibition results demonstrate that the anti-CD4 MAb, P1, exhibits broad inhibitory activity against HIV-1 clade A, C, D, and E isolates, based on a reduction of HIV-1 p24 antigen concentrations compared with untreated controls and controls treated with an irrelevant MAb. Inhibitory concentrations of P1 (100 μg/mL) are well within the range of anti-CD4 MAbs that have been infused into patients and maintained for weeks at a time with no deleterious effects [10]. Interestingly, little to no inhibition activity was observed with the primary HIV-1 clade B isolate BZ167. This lack of inhibitory activity against the BZ167 isolate did not appear to reflect infectious titer. Other clades that exhibited titers higher than or equivalent to that of BZ167 were efficiently inhibited by P1. If the input titer of BZ167 was reduced to 10 $TCID_{50}$, P1 was capable of neutralizing infectivity (data not shown). The reasons for the inability of P1 to effectively inhibit this primary HIV-1 clade B isolate at titers at which other non–clade B isolates are neutralized is unknown. It is possible that BZ167 is not representative of other clade B primary isolates. Indeed, a second clade B primary isolate, designated A284, along with other non–clade B isolates, were inhibited from infecting PBMC by P1, similar to the data presented in table 1 (data not shown). This suggests that BZ167 is not representative of other clade B primary isolates. These data suggest that BZ167 may infect PBMC without the need for a CD4 interaction or may interact with a CD4 epitope not recognized by P1 and different from the CD4 epitope used by the other primary isolates of HIV-1.

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Human Immunodeficiency Virus Proviral DNA from Peripheral Blood and Lymph Nodes Demonstrates Concordant Resistance Mutations to Zidovudine (Codon 215) and Didanosine (Codon 74)

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Genotypes that confer drug resistance were evaluated in human immunodeficiency virus (HIV) proviral DNA obtained from peripheral blood mononuclear cells (PBMC) and lymphoid tissue at baseline and after 8 weeks of therapy with zidovudine alone or in combination with didanosine from 22 patients (8 zidovudine-naive and 14 zidovudine-experienced). There was evidence of zidovudine resistance at codon 215 in 27.3% (6/22) of patients. All 20 patients evaluable for codon 74 (site of didanosine resistance) had virus that remained wild type during the 8-week study period. When HIV proviral DNA from PBMC was compared with that from lymphoid tissue, 94.7% (18/19) of evaluable samples were concordant at codon 215 at baseline, while 85.7% (12/14) were concordant at week 8. Resistance in PBMC (but not in lymphoid tissue) developed in 1 of 8 zidovudine-naive patients; an increased proportion of resistant strains in PBMC (but not in lymphoid tissue) was observed in 2 of 14 zidovudine-experienced patients. These results suggest high concordance for drug resistance mutations in HIV proviral DNA from blood and lymph node tissue.

Drug-resistant human immunodeficiency virus type 1 (HIV-1) isolates have been detected in clinical samples from patients receiving all of the currently approved antiretroviral agents. These isolates are detected in peripheral blood after variable lengths of time, depending on the host immune status, virus load, and antiviral potency of the drug or drug combination used for treatment [1]. For nucleoside antiviral agents, drug-resistant virus is detected first in the plasma viral RNA or virus isolates cocultured from peripheral blood mononuclear cells (PBMC), followed by gradual replacement of the PBMC proviral DNA with mutant virus [2]. The picture is less clear for HIV protease inhibitors, with one recent report describing simultaneous detection of drug resistance mutations in the plasma HIV RNA and proviral DNA from PBMC [3].

There are isolated reports comparing viral drug resistance genotypes in blood with those obtained from tissues from HIV-infected patients receiving therapy. The relationship of emergence of drug-resistant virus in tissue versus blood has not been carefully evaluated in clinical trials. Discordant genotypes have been reported for zidovudine-resistant virus in peripheral blood and brain tissue samples obtained at autopsy [4]. No drug resistance data have been presented for sequential samples of blood and lymphoid tissue obtained from patients while receiving antiretroviral therapy. Here we describe the results of genotypic drug resistance analyses of HIV proviral DNA from PBMC and lymph node samples obtained at baseline and after 8 weeks of randomly assigned antiretroviral therapy.

Methods

The Division of AIDS Treatment Research Initiative 003 study enrolled 35 HIV-infected persons between June 1992 and March 1993, and 32 completed the 8-week study [5, 6]. Sixteen antiretroviral-naive patients with CD4 cell counts $\geq 350/mm^3$ were randomly assigned to either zidovudine at 500 mg/day or no treatment; the 8 untreated patients were excluded from this analysis. Sixteen patients with ≥ 26 weeks of continuous zidovudine and CD4 cell counts $\geq 250/mm^3$ were randomly assigned to either continued zidovudine monotherapy or zidovudine plus didanosine for 8 weeks; of these patients, 2 were excluded from this analysis because of unavailability of samples.

Specimen collection and processing. Mononuclear cells were obtained from peripheral blood and axillary or cervical lymph nodes, and pelleted cells were frozen at −70°C until use. The specimen collection and processing methods and the consistency

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Human experimentation guidelines of the US Department of Health and Human Services were followed in the conduct of this study. The protocol was approved by the institutional review boards of each participating site. Informed consent was obtained from all study participants.

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of histopathologic and virus burden measurements in a given individual's lymph nodes, regardless of source, have been described previously [5, 6].

Analysis of genotypic resistance in peripheral blood and lymph node mononuclear cells by differential polymerase chain reaction (PCR). Lysates corresponding to 10^5 cells in 50 μL were mixed with 30.5 μL of diethyl pyrocarbonate (DEPC) H_2O, 5 μL of PCR 10× buffer (Perkin-Elmer Cetus, Norwalk, CT), 5 μL (0.25 μg) of primer A (5′-TTGGTTGCACTTTAAATTTTCCCATTAGTCCTATT-3′), 5 μL (0.25 μg) of NE1 primer (5′-CCCACTAACTTCTGTATGTCATTGACAGTCCAGCT-3′), 4 μL of 25 m*M* $MgCl_2$, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus) for the first PCR reaction. The PCR profile is as follows: 94°C for 1 min; 30 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min; and then 72°C for 10 min.

Codon 215 differential PCR [*7*]. Master mixes for the 215 mutation and wild type codon were set up as follows: 10 μL of manufacturer's 10× PCR buffer, 3 μL of 25 m*M* $MgCl_2$, 10 μL of dNTPs, and 3 μL of forward primer B (5′-GGATGGAAAGGATCACC-3′) and the 215W primer (5′-ATGTTTTTTGTCTGGTGTGGT-3′) or 215M primer (5′-ATGTTTTTTGTCTGGTGTGAA-3′). Next, 10 μL of the diluted A/NE1 first PCR product was added to each of the 215 mixes along with 56 μL of H_2O and 0.5 μL of Taq polymerase. The cycling profile for both reactions was as follows: 94°C for 5 min; 20 cycles of 94°C for 1 min, 48°C for 30 s, and 72°C for 30 s; and then 72°C for 10 min. PCR product (10 μL) was analyzed on an agarose gel for presence or absence of the specific product.

Codon 74 differential PCR [*8*]. Master mixes for the 74 mutation and wild type codon were set up as follows: 10 μL of manufacturer's 10× PCR buffer, 3 μL of 25 m*M* $MgCl_2$, 10 μL of dNTPs, and 3 μL of forward primer A (5′-TTCCCATTAGTCCTATT-3′) and the 74W primer (5′-AAGTTCTCTGAAATCTACTTA-3′) or 74M primer (5′-AAGTTCTCTGAAATCTACTTC-3′). Next, 10 μL of the diluted A/NE1 first PCR product was added to each of the 74 mixes along with 56 μL of H_2O and 0.5 μL of Taq polymerase. The cycling profile for both reactions was as follows: 94°C for 5 min; 20 cycles of 94°C for 1 min, 48°C for 30 s, and 72°C for 30 s; and then 72°C for 10 min. PCR product (10 μL) was analyzed on an agarose gel for the presence or absence of the specific product.

All mixtures were serially diluted to determine that a mixture of genotypes was present and to avoid artifact due to template overload. Previous studies with defined mixtures of wild type and mutant virus at codon 215 have shown that differential PCR will yield a wild type result if <10% of the virus is mutant, while mixtures with 10%–60% mutant virus will be classified as mixed and mixtures with >60% mutant virus will be classified as resistant.

Phosphorylated zidovudine levels. Phosphorylated zidovudine levels were measured on lymphoid tissue cell lysates by RIA as described previously [9].

Statistical analysis. Confidence intervals (CIs) and upper bounds for patients with resistant virus were computed by use of exact methods [10]. Separate logistic regression models were used to evaluate the association between likelihood of developing resistance and virus load, prior zidovudine use, and intracellular zidovudine phosphorylation. Logistic regression analysis was conducted by use of the SAS LOGISTIC procedure [11].

Results

Patients. Samples from 22 study participants were analyzed for this study. Most patients (20/22) were men and had male homosexual contact as their HIV risk factor (17/22). For the 8 zidovudine-naive and 14 zidovudine-experienced patients, the mean age was 35 and 37 years, and the mean CD4 cell count was 655 and 414 cells/mm^3, respectively. The zidovudine-experienced patients had been receiving the drug for an average of 26.8 months, and the mean baseline phosphorylated zidovudine level in lymphoid tissue cells was 20.8 pmol/10^6 cells. The baseline drug resistance analysis included PBMC and lymphoid tissue samples from 8 zidovudine-naive patients and 13 zidovudine-experienced patients. The week 8 drug resistance analysis included PBMC and lymphoid tissue samples from 8 patients who were zidovudine-naive at enrollment and then received 8 weeks of zidovudine and from 6 patients who were zidovudine-experienced and then received 8 weeks of zidovudine and didanosine.

Codon 215 (zidovudine resistance) mutations in peripheral blood and lymphoid tissues. The majority of samples had detectable signal. All 35 PBMC samples and 34 of 35 lymphoid tissue samples were evaluable for codon 215 mutations.

During the 8-week study, 27.3% of the participants (95% CI, 14%–45%) showed some evidence of resistance at codon 215 (table 1). Of the 8 participants who were zidovudine-naive at entry, 1 showed resistance at baseline; a second who had wild type virus at entry had virus that had become mixed after 8 weeks (95% CI, 3%–37%). In the former participant, it is not known whether primary infection was with a drug-resistant virus or if there was unacknowledged prior exposure to zidovudine. In the latter participant, this change was apparent only from PBMC samples, with all tissue samples showing wild type virus. Of the 14 participants with prior zidovudine use, 2 showed increased resistance (95% CI, 5%–34%). One patient, whose virus was wild type at entry, had virus that had become mixed by 8 weeks; another whose virus had been mixed at entry had resistant virus by 8 weeks. In both patients, these changes in resistance were evident only from PBMC samples.

Table 1. Summary of codon 215 resistance analysis.

	Lymphoid tissue result		
Blood result	Wild type	Mixed	Resistant
Baseline*			
Wild type	15	1	0
Mixed	0	1	0
Resistant	0	0	2
Week 8			
Wild type	9	0	0
Mixed	1	1	0
Resistant	0	1	2

* At baseline, 1 peripheral blood mononuclear cell sample and 2 lymphoid tissue samples (from 3 different patients) were not available for analysis.

Results of the codon 215 analysis were concordant in 94.7% (18/19 evaluable persons; 95% CI, 82%–99%) of the study participants at baseline. At week 8, 85.7% (12/14 evaluable participants; 95% CI, 66%–95%) were concordant. Of the discordant samples, 1 was wild type in tissue and mixed in blood; 1 was mixed in tissue and wild type in blood; and 1 was mixed in tissue and resistant in blood. No samples were wild type in one compartment and resistant in the other.

Codon 74 (didanosine resistance) mutation. All 20 evaluable patients' virus remained wild type at codon 74 during the 8 weeks of the study (the 95% upper confidence limit for the percentage resistant at codon 74 is 13%).

Parameters associated with zidovudine resistance. There was no association between baseline virus load (HIV DNA or RNA), duration of previous zidovudine exposure, or intracellular phosphorylation of zidovudine and the presence of zidovudine-resistant HIV strains in this small group of patients (data not shown).

Discussion

Zidovudine resistance has been associated with the development of mutations at codons 41, 67, 70, 215, and 219 for the HIV reverse transcriptase gene [12, 13]. The codon 215 mutation is required for development of high-level phenotypic drug resistance, typically in combination with mutations at either codon 41 or 67 and 70. These mutations arise after weeks to months of therapy, and high-level drug resistance (zidovudine $IC_{50} > 1\ \mu M$) usually begins to emerge after 6 months of zidovudine monotherapy. In this study, 27.3% of study participants had genotypic evidence of zidovudine resistance, with mutations detected at codon 215. Three patients showed evidence of an increase in the fraction of mutant virus detected in the HIV proviral DNA during the 8 weeks of the study. The majority of paired samples were concordant for codon 215 mutations in PBMC and lymph node cells. Discordant samples occurred for 3 sample pairs and always involved mixtures. These discrepancies could result from technical artifacts due to the difficulty of resolving mixtures by differential PCR or could suggest subtle differences in rates of emergence of mutant virus in blood versus tissue compartments in these patients. Since proviral DNA was evaluated, the relationship of actively replicating virus in lymph node tissue could not be related to circulating HIV RNA in this study.

Didanosine resistance has been associated with mutations at codons 65, 74, and 184 in patients receiving didanosine monotherapy and, more recently, with a combination of mutations at codons 62, 75, 77, 116, and 151, which produce resistance to zidovudine, didanosine, and zalcitabine, in patients receiving combination therapy with zidovudine and didanosine [8, 14]. The codon 74 mutation is seen most frequently with didanosine monotherapy and has been detected in 65% of patients after 1 year of therapy. Combination therapy with didanosine and zidovudine decreases detection of the codon 74 mutations to <4% of patients after 1 year of therapy [15]. A minority of patients receiving didanosine and zidovudine therapy develop multinucleoside resistance via the pathway using the 151 mutation. All patients who entered the study had virus that was wild type at codon 74 and remained wild type during the 8 weeks of combined therapy with zidovudine and didanosine. The low frequency of new resistance mutations seen in this study is most likely due to the short period of follow-up, although changes at other codons in the reverse transcriptase gene might have been detected if the samples had been sequenced.

Previous studies evaluating drug resistance genotypes in peripheral blood and brain tissue have suggested different populations of zidovudine-resistant virus in these tissue compartments [4]. This could be related to differential cell tropism by virus strains or differential drug exposure because of the blood-brain barrier. In this study, the great majority of sample pairs from PBMC and lymph node tissue were concordant at codons 74 and 215, suggesting that similar drug-resistant populations were present in both compartments. All discordant samples involved mixtures of sensitive and resistant virus. Further studies will be needed to characterize the time course and potential tissue compartment heterogeneity of drug-resistant HIV-1 in blood, lymphoid tissue, the central nervous system, and semen. These data suggest that PBMC- and lymph node–associated HIV-1 have comparable drug resistance genotypes in patients treated with nucleoside antiretroviral agents.

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Effect of Granulocyte Colony-Stimulating Factor (G-CSF) in Human Immunodeficiency Virus–Infected Patients: Increase in Numbers of Naive CD4 Cells and CD34 Cells Makes G-CSF a Candidate for Use in Gene Therapy or to Support Antiretroviral Therapy

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The potential of granulocyte colony-stimulating factor (G-CSF) to mobilize CD4 cells and/or CD34 cells for use in gene therapy or to support antiretroviral therapy was examined. Ten human immunodeficiency virus–infected patients were treated with G-CSF (300 μg/day) for 5 days. Numbers of CD4 and CD34 cells were measured. To examine the numbers of naive and memory type CD4 cells, CD4 cell coexpression of CD45RA and CD45RO was measured. Functionality of mobilized CD4 cells was examined by use of the proliferation assay and interleukin-2 ELISA. The number of CD34 cells increased from 1.50 to 20.01/μL ($P < .002$). The CD4 cell count increased from 236 to 452/μL ($P < .002$). The CD45RA/CD45RO ratio increased from 0.50 to 0.57 ($P < .03$). Mobilized CD4 cells were functionally intact. In conclusion, G-CSF induced increases in numbers of CD34 cells and CD4 cells in HIV-infected patients. Furthermore, the fraction of naive CD4 cells increased. These findings have implications for the design of immunotherapy or gene therapy protocols.

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor that increases the numbers of circulating hematopoietic progenitor cells (CD34 cells) and CD4-positive T lymphocytes (CD4 cells) in human immunodeficiency virus (HIV)–infected patients [1–4]. In the present study, we examined whether G-CSF might be useful in gene therapy or to support antiretroviral treatment. The G-CSF–induced increase in numbers of CD34 cells and CD4 cells in HIV-infected patients was examined. CD4 cell coexpression of CD45RA and CD45RO was used to characterize mobilized CD4 cells as naive and memory cells, respectively. Furthermore, the functionality of mobilized CD4 cells was examined.

Patients and Methods

Patients and study design. Ten HIV-infected patients were included (table 1). The patients received G-CSF (300 μg of filgrastim/day; Amgen, Thousand Oaks, CA) administered subcutaneously once a day for 5 consecutive days. Baseline variation was determined with blood samples collected on 4 days before G-CSF treatment (days −6 to −3). To determine the effect of G-CSF

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The protocol was approved by the local ethical committee (KF 02-194/96). Informed consent was obtained from all patients after the nature and consequences of the study had been fully explained.

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Table 1. Entry data of 10 HIV-positive patients in study of G-CSF treatment.

Patient no.	Sex	Age (years)	CD4 cell count/μL	Virus load (copies/mL)	Clinical symptoms	Antiviral treatment
1	M	53	269	1867	VZV	D4T, ddI, saquinavir, ritonavir
2	M	53	235	<200	HIV wasting	3TC, ddI, indinavir
3	M	48	103	357	Kaposi's sarcoma	3TC, D4T, indinavir
4	M	50	242	<200	VZV	AZT, 3TC, indinavir
5	M	30	288	67,308	Esophageal candidiasis	AZT, 3TC
6	M	31	246	15,348	Multiple bacterial pneumonias	3TC, D4T, indinavir
7	M	59	323	42,970	*Pneumocystis carinii* pneumonia	AZT, 3TC, saquinavir
8	M	42	300	<200	VZV	AZT, 3TC, indinavir
9	M	49	237	<200	Pulmonary tuberculosis	AZT, 3TC, indinavir
10	M	65	115	<200	Oral candidiasis	AZT, 3TC, indinavir
Mean		48	236	12,885		
Range		30–65	103–323	<200–67,308		

NOTE. VZV, varicella-zoster virus. D4T, stavudine; ddI, didanosine; 3TC, lamivudine; AZT, zidovudine.

treatment, blood samples were collected during G-CSF treatment (days 0–4) and on the following 4 days (days 5–8). Blood collected in tubes containing EDTA was used to obtain a full blood cell count and for flow cytometry. On days −6, −3, 0, 4, and 8, additional blood samples were drawn into tubes containing heparin to obtain peripheral blood mononuclear cells (PBMC) by means of density gradient centrifugation.

Flow cytometry. Flow cytometric analyses were done as described [4]. The fluorescence of 5000 cells (lymphocyte subsets) or 50,000 cells (CD34 and isotype controls) was measured. To determine the absolute numbers of CD34 cells and lymphocytes in peripheral blood, the percentage of the cells expressing CD34 was multiplied by the white blood cell count, while the percentage of cells expressing CD3, CD4, and CD8 in a lymphocyte gate was multiplied by the lymphocyte count. The following combinations of monoclonal antibodies were used to determine phenotypes: isotype control; CD3 (Leu-4, SK7), CD4 (Leu-3a, SK3), CD8 (Leu-2a, SK1); CD4 (Leu-3a, SK3), CD45RA (Leu-18, L48), CD45RO (Leu-45RO, UCHL-1); CD4 (Leu-3a, SK3), CD25 (2A3), CD69 (Leu-23, L78); CD34 (anti-HPCA-2). All monoclonal antibodies were purchased from Becton Dickinson (San Jose, CA).

Proliferation assay. On days −6, −3, 0, 4, and 8, PBMC were used for proliferation assay, done essentially as described [5]. Cell cultures were prepared in microtiter plates (Nunc, Roskilde, Denmark) containing 5×10^4 PBMC/well. Culture medium was RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and a mixture of penicillin, streptomycin, and gentamicin. To stimulate cultures, 5 μg/mL phytohemagglutinin (PHA; Sigma, St. Louis) was added. In each case, control experiments without stimulation were done. Results are reported as proliferation response to PHA minus response to plain culture medium. All results are medians of values from quadruplicate cultures.

Interleukin-2 (IL-2) ELISA. PBMC were used for IL-2 ELISA. Briefly, cell cultures were prepared in microtiter plates (Nunc) containing 10^5 PBMC/well. To the cultures, 4 μg/mL PHA (Sigma) was added. In each case, control experiments without stimulation were done. After 3 days in a cell incubator, the culture supernatants were harvested. Predicta IL-2 kit (Genzyme, Cambridge, MA) was used to quantitate IL-2 production. The assay was done according to the instructions of the manufacturer. Results are reported as IL-2 production in response to PHA stimulation minus response to plain culture medium. All results are medians of values from duplicate cultures.

Measurements of virus load. To determine virus load, quantification of viral RNA in plasma was done with a polymerase chain reaction quantitative kit (Amplicor Monitor HIV-1 PCR; Roche, Branchburg, NJ) according to the instructions of the manufacturer. Plasma was obtained after centrifugation at 400 *g* for 7 min, harvested, and immediately stored at −70°C until use.

Statistical analysis. Data points obtained from multiple experiments were reported as means (±SEs). Significance levels were determined by Student's *t* test analysis. For comparisons within the group, we used a paired-sample *t* test.

Results

G-CSF effect on the absolute numbers of CD34 and CD4 cells. The absolute number of circulating CD34 cells increased significantly during G-CSF treatment (figure 1A). On day 0, the mean number of CD34 cells was 1.50/μL (±0.11). On day 2, the number of CD34 cells had increased significantly ($P < .04$), and the mean number of CD34 cells peaked on day 5 after initiation of G-CSF treatment, at 20.01/μL (±0.83, $P < .002$). By day 8, the number of CD34 cells had almost returned to baseline (figure 1A). In contrast, there was no significant variation in the number of CD34 cells before G-CSF treatment. The number of CD34 cells increased in all 10 patients. The increase in number of CD34 cells was partly due to an increase in white blood cell count from 5.97×10^9/L (±0.48) to 24.45×10^9/L (±2.63).

Before G-CSF treatment, there was only minor variation in the mean CD4 cell count (from 236 to 279/μL, figure 1B). On day 0, the mean number of CD4 cells was 236/μL (±23). On day 1, the CD4 cell count had increased significantly ($P < .02$), and the number of CD4 cells peaked on day 3 after

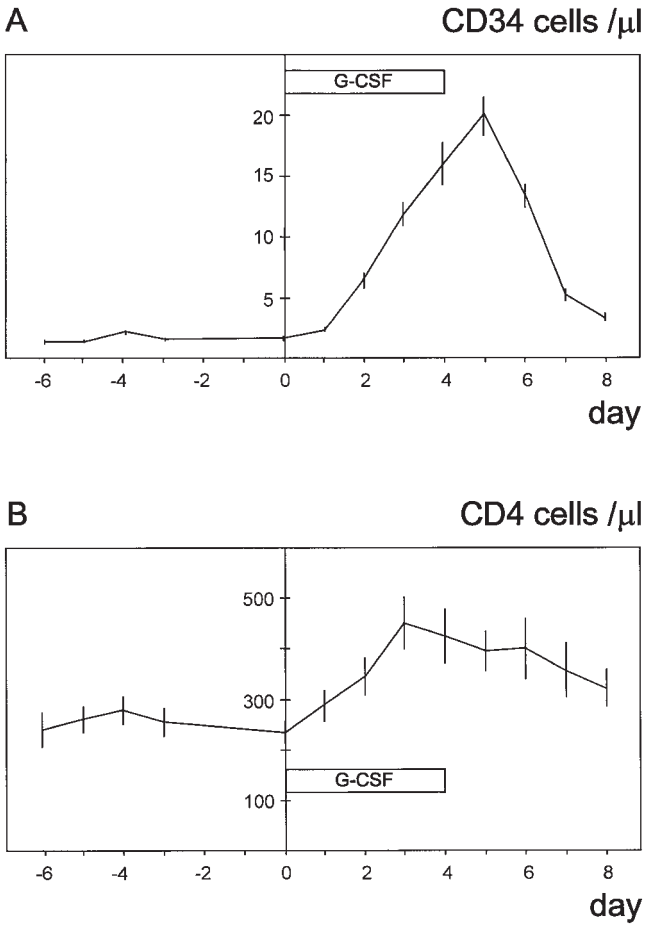

Figure 1. Numbers of circulating CD34 cells (**A**) and CD4 cells (**B**) (cells/μL, mean ± SE) in 10 HIV-positive men before (days −6 to −3), during (days 0–4), and after (days 5–8) G-CSF treatment.

initiation of G-CSF treatment, at 452/μL (±52, $P < .002$). Interestingly, although there was a decrease in CD4 cell count during the rest of the study period, it did not return to baseline (figure 1B). Thus, the CD4 cell count was 323/μL on day 8 (±38, $P < .02$). For all 10 patients, we found the CD4 cell count to increase by at least 100/μL. The increase in CD4 cell count was mainly due to an increase in lymphocyte count from 2.26×10^9/L (±0.38) to 3.28×10^9/L (±0.48).

Increase in CD45RA/CD45RO ratio. To evaluate the numbers of naive and memory-type CD4 cells, we measured CD4 cells coexpressing CD45RA and CD45RO. On day 0, the mean percentage of CD4 cells expressing CD45RA and CD45RO was 31.2% (±3.6) and 68.1% (±3.7), respectively. CD45RA and CD45RO values on day 4 were 33.4% (±3.9) and 65.8% (±4.2). The increase in percentage of CD45RA cells was significant, as was the decrease in CD45RO cells ($P < .01$). The mean CD45RA/CD45RO ratio was 0.50 on day 0, and the ratio increased significantly to 0.57 on day 4 and 0.61 on day 8 ($P < .03$). In contrast, no significant differences were found to exist between ratios on day 0 and day −6 or −3. Although the percentage of CD45RO-positive cells decreased, there was an increase in the absolute number of both CD45RA- and CD45RO-positive cells. Thus, on day 0, the mean numbers of CD4 cells expressing CD45RA and CD45RO were 79 and 155/μL. On day 4, the mean numbers of CD4 cells expressing CD45RA and CD45RO were 154 and 267/μL.

No evidence of peripheral proliferation. To evaluate if CD4 cells were generated by peripheral proliferation, we measured CD4 cells coexpressing the activation antigens CD69 and CD25. On day 0, 12.4% (±5.5) of CD4 cells expressed CD69, and on day 4 this fraction was reduced to 9.3% (±5.6) ($P < .04$). In contrast, no differences were found between values for day 0 and day −6, −3, or 8. Furthermore, on day 0 we found that 13.0% (±3.6) of CD4 cells coexpressed CD25. This fraction was not different from the fractions we found on any other day.

Functionality of mobilized CD4 cells. The functionality of CD4 cells was examined by use of a proliferation assay. All patients responded to PHA, with a mean proliferative response of 110.7×10^3 cpm on day 0. There were no significant differences between proliferative responses on day 0 and on any other day. In addition, the functionality of the CD4 cells was evaluated by IL-2 production. On day 0, PBMC from 7 patients produced IL-2 in response to stimulation with PHA (mean, 33 pg/mL [±14]). On day 4, PBMC from 8 patients produced IL-2 in response to PHA (mean, 81 pg/mL [±59]). However, no significant differences in IL-2 production in response to PHA were found. Thus, the CD4 cells mobilized by G-CSF treatment appear to have normal functionality.

Virus load during G-CSF therapy. Virus load during G-CSF treatment was tested by use of HIV RNA polymerase chain reaction. In 6 patients, we detected an increase in copy number per milliliter, in 3 patients the copy number was <200 on day 0 as well as on day 4, and in 1 patient there was a decrease in virus load. On day 0, the mean copy number was 12,885/mL (±7421), and on day 4 it was 16,993 (±11,116). Thus, we found a minor increase in virus load. This increase, however, was not significant ($P = .47$).

Discussion

This study demonstrates that G-CSF increases both CD34 and CD4 cells in HIV-infected persons. Furthermore, we found an increase in percentage of naive CD4 cells. These data make G-CSF a candidate for use in gene therapy or to support antiretroviral therapy.

Three events might explain the rise in CD4 cell count: differentiation of CD34 cells, peripheral proliferation, and redistribution of CD4 cells from the lymphatics. Studies in mice have shown that naive CD4 cells exported from the thymus express CD45RA. By contrast, memory CD4 cells derived from peripheral proliferation express CD45RO [6]. However, interconversion between CD45RA and CD45RO has been demonstrated [7], suggesting that isoform expres-

sion may not reflect a unidirectional maturation from naive to memory type cells. Thus, the rise in CD45RA/CD45RO ratio does support differentiation of CD34 cells but does not provide definite proof. The rapid increase in CD4 cell count could indicate that G-CSF had an effect on CD34 cells in the thymus that were already committed to T cell generation.

A recent study concluded that expansion of peripheral lymphocytes in vivo is antigen-driven [8]. Increased CD69 and CD25 expression by lymphocytes activated by antigens has been documented [9, 10]. We did not detect increased expression of either CD69 or CD25. Thus, peripheral proliferation does not seem to explain the increase in CD4 cell count. Finally, redistribution of CD4 cells from the lymphatics might explain the rise in CD4 cell count. The rapid increase in CD4 cell count does support redistribution as being partly responsible. Furthermore, G-CSF has been shown to alter cytokine production in blood, which might result in lymphocyte redistribution as well [11]. However, if redistribution was the sole explanation for the rise in CD4 cell count, the switch toward the naive phenotype would not be expected. Thus, further studies to examine the effect of G-CSF on CD34 cell differentiation are needed.

HIV infection is characterized by a progressive decline in CD4 cell count. A recent study of the effects of protease inhibitors demonstrated that after protease inhibitor therapy, naive CD4 cells increased only if they were present before initiation of therapy [12]. The generation and maintenance of a diverse T cell repertoire are central requirements for immune competence, and it therefore remains uncertain whether even highly efficient antiretroviral therapies will result in restoration of the immune system once damaged. Recently, reports of cytomegalovirus retinitis in HIV-infected patients with CD4 cell counts >195 after initiation of protease inhibitor therapy has been published [13]. Such cases of opportunistic infections in patients with high CD4 cell counts could be due to a skewness in CD45RA/CD45RO ratio. In the present study, an increase, although modest, was found in the absolute number of naive CD4 cells during 5 days of G-CSF treatment. It cannot be excluded that a more pronounced effect could be found if HIV-infected patients were treated with G-CSF for a longer period of time. If that is the case, G-CSF might be used in combination with protease inhibitors to maintain a diverse T cell repertoire.

Gene therapy has been suggested as a possible treatment of HIV infection [14, 15]. The recovery of high yields of functionally unimpaired T lymphocytes or progenitor cells is a mandatory prerequisite for such approaches. The present study demonstrates that G-CSF can be used to mobilize functional CD4 cells as well as CD34 cells prior to genetic intervention. Furthermore, it cannot be excluded that the increase in CD4 cell count is partly due to differentiation of CD34 cells. These findings have implications for the design of gene therapy protocols.

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Apparent Enhancement of Perinatal Transmission of Human Immunodeficiency Virus Type 1 by High Maternal Anti-gp160 Antibody Titer

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The presence of antibodies able to enhance infection in vitro in sera from human immunodeficiency virus (HIV)-1–infected patients raises the possibility that antibodies exert a deleterious activity during natural infection. The anti–HIV-1 humoral response and plasma HIV-1 RNA were measured in a cohort of 98 infected mothers, included in the French Prospective Study on Pediatric HIV Infection, 49 of whom transmitted HIV to their children. Transmission from mother to child was associated with antibody responses to the envelope gp160 ($P = .009$ for serum dilution of 1/400) and to a highly conserved domain of the transmembrane glycoprotein ($P = .055$ for serum dilution of 1/400) and with plasma HIV-1 RNA levels ($P < .0001$). Multivariate logistic regression indicated that a high anti-gp160 response and a high plasma virus load are independent risk factors for perinatal transmission of HIV-1 (odds ratio, 3.4; 95% confidence interval, 1.1–9.9 for anti-gp160; odds ratio, 2.8; 95% confidence interval, 1.6–5.0 for virus load).

The role of the humoral immune response in human immunodeficiency virus type 1 (HIV-1) infection, whether protective or harmful, is a matter of debate. Although neutralizing antibodies are detectable during the chronic phase of infection in most seropositive persons, antibodies capable of enhancing viral infection are also frequently found in sera from HIV-1–infected persons [1]. Antibody-mediated enhancement of HIV-1 infectivity in vitro has been correlated with clinical progression to disease in HIV-1–infected patients [2]. Moreover, an association between the antibody responses to certain epitopes of HIV-1 envelope in the first months after seroconversion and progression to disease was found in HIV-1–infected adults [3].

We thought that an appropriate situation in which to address the role of HIV-enhancing antibodies in vivo would be the transmission of infection from mother to child. During pregnancy of HIV-1–infected women, anti-viral IgG may affect virus transmission by interacting with virus in either the maternal or the fetal circulation after passive transfer of antibodies to the fetus during the last months of pregnancy. At present, data concerning the relationship between maternal antibodies and risk of mother-to-child transmission of infection are controversial. HIV-1–neutralizing antibodies or antibodies directed against the principal neutralizing domain of the HIV-1 envelope gp120, the V3 loop, have been correlated with a lower transmission [4–6]. However, such correlations were not confirmed by other groups [7–9], and two studies pointed out a direct correlation between antibodies directed to V3 epitopes and higher perinatal transmission [10, 11].

We decided to analyze the anti–HIV-1 humoral response at delivery in a cohort of both Caucasian and African mothers in relation to materno-fetal transmission. Our study was focused on antibodies against the viral envelope, since these have been most directly implicated in neutralization or enhancement phenomena. The use of the envelope precursor, the gp160, was aimed at obtaining a global evaluation of the anti-envelope humoral response and reducing the bias due to the variability of the gp120 and, in particular, the immunodominant V3 domain. To test the possibility that disparate observations were due to the complexity of the antibody response against neutralizing and enhancing epitopes on the envelope, we dissected the humoral response by selecting peptides corresponding to two highly conserved antigenic domains of the extracellular gp120 (SU) and the transmembrane gp41 (TM) glycoproteins of HIV-1. In particular, we used a peptide corresponding to the principal immunodominant domain (PID) of the TM, which is the most conserved antigenic domain of the envelope of HIV-1 [12].

Materials and Methods

Study population. This case-control study included 98 mother-infant pairs involving HIV-1–infected mothers whose infants are enrolled in the French Prospective Study on Pediatric HIV Infection [13]. The present study concerns the subgroup of mothers of the infants enrolled in the 41 centers located in the Paris area that

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send blood specimens to the virology laboratory of the Hôpital Necker-Enfants Malades. Forty-nine women transmitted HIV-1 infection to their infants (''transmitters''), while 49 women did not (''nontransmitters''). Pairs in which infected infants were born between December 1988 and March 1994 were included if blood samples of the mother were available. Nontransmission controls were matched for the nearest time of entry in the French Cohort with the transmission cases. Seventy-four mothers (34 nontransmitters and 40 transmitters) were of Caucasian origin, and 24 mothers (15 nontransmitters and 9 transmitters) were of African origin.

Twelve of 49 infected children developed an early and severe form of disease, defined by the following criteria occurring before 18 months of age: classification in C category (1994 CDC classification for children) for 5 children, severe lymphopenia (category 3) for 5 children, death for 2 children.

Maternal plasma samples, in which anti-HIV antibodies, p24 antigenemia, and HIV-1 RNA were measured, were collected at delivery or in the first week after delivery.

Antigens. Various antigens were used in this study. Peptides were the SUpep peptide (obtained from ANRS, Paris) from the C-terminal sequence of the SU (HIV-1_{LAI} aa 491–516: YKVVKIEPLGVAPTKAKRRVVQREKR) and the PID peptide (HIV-1_{LAI} aa 598–611: LGIWGCSGKLICTT; Neosystem, Strasbourg, France), cycled between cysteines to simulate the natural loop conformation of the principal immunodominant domain of the TM.

The p25-LAI protein, produced in *Escherichia coli,* corresponds to the HIV-1 capsid, referred to in this study as p24. The gp160s-MN/LAI, produced by recombinant vaccinia virus–infected BHK cells, corresponds to a soluble form of Env precursor. Recombinant proteins were provided by Transgene (Strasbourg, France).

All antigens were tested by use of a panel of uninfected or HIV-1–infected sera, to optimize ELISA conditions. As a control, an irrelevant 18-aa peptide from the ICAM protein of rat (p313) was used.

ELISA. Microtiter plates (Immulon 2; Dynatech, Chantilly, VA) were coated with peptides and proteins in 100 μL of 0.1 *M* sodium carbonate buffer, pH 9.6, at 4°C overnight. The peptides were used at a concentration of 5 μg/mL, the p24 protein at 1.5 μg/mL, and the gp160s protein at 0.25 μg/mL. The plates were blocked with 200 μL of 5% fetal calf serum (FCS) in PBS for 2 h at room temperature. After three washes with PBS containing 0.5% Tween 20 (washing buffer), 100 μL of mothers' sera diluted in PBS with 5% FCS and 0.5% Tween 20 (dilution buffer) was added to the wells and incubated for 2 h at room temperature. After five washes, a peroxidase-conjugated anti-human IgG Fc (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted to 0.8 μg/mL in dilution buffer was added for 1 h at room temperature. After five washes, the reaction was visualized with 0.4 mg/mL ABTS (Sigma, St. Louis) in 0.6% acetic acid, pH 4.7, containing 0.012% hydrogen peroxide. After 30 min, optical density was measured at 405 nm (Multiskan RC; Labsystems, Helsinki). Assays were done in duplicate and results expressed as means. In each microplate, wells coated with the PID peptide and incubated with a reference pool of HIV-1–positive human sera diluted at 1/1350 were included. Normalization of the results in relation to the reference serum allowed a direct comparison of ELISA results obtained at different times and on separate plates. Moreover, optical density values obtained with HIV-1 antigens were corrected by subtraction of the background level observed using the p313 peptide.

Quantification of plasma virus load. HIV-1 RNAs in plasma were titrated by reverse transcription followed by polymerase chain reaction (RT-PCR; AMPLICOR HIV Monitor test; Roche Diagnostic Systems, Nutley, NJ). For African mothers, extra primers (add-in primers), provided by Roche, were used to improve quantification of HIV-1 clades other than B. The add-in primers were designed to amplify the same region of the HIV-1 *gag* gene that is amplified by the standard primers of the AMPLICOR HIV Monitor test. The add-in primers were added to the standard primers in the same PCR reaction. This method has been validated in several laboratories, and in particular by our laboratory in experiments on 50 well-characterized non-B HIV-1 isolates (data not shown). As plasmas were collected on sodium heparin, which inhibits the test, measurements were done on viral pellets obtained by ultracentrifugation.

p24 antigenemia was measured by use of kits from Abbott Laboratories (Abbott Park, IL) or Organon Teknika (Baxtel, The Netherlands).

Statistical analysis. ELISA (antibody titers) and RT-PCR (plasma virus load) results were compared between groups by nonparametric Mann-Whitney test. To assess the role of the humoral response and potential confounding factors on the risk of vertical transmission, univariate and multivariate stepwise logistic regression analyses were used (SPSS software; SPSS, Chicago). Antibody responses were considered as continuous variables. The confounding maternal factors that were considered were as follows: CDC stage (A, B-C, unknown), origin (African, Caucasian), CD4 cell counts ($\leqslant$350, 351–500, $>$500 cells/mm^3, unknown), p24 antigenemia (positive, negative), and plasma HIV RNA taken as a continuous variable. We also entered in the model the interaction terms between each confounding factor (CDC stage, ethnic origin, CD4 cell counts, p24 antigenemia, plasma HIV RNA) and the antibody response against each antigen studied. Only interaction terms exhibiting a *P* value below the commonly used threshold of .20 were included in the final model.

Results

Antibody response against HIV-1 antigens. Sera from 49 nontransmitting mothers and 49 sera from transmitting mothers were tested for reactivity against SU and PID peptides and p24 and gp160s proteins. Both groups showed a wide diversity in the level of antibody responses. Serum dilutions that produced absorbance values in the dynamic range for most sera (1/400 and 1/800 for p24, gp160s, and PID and 1/200 and 1/400 for SUpep) were used for the comparison of transmitter and nontransmitter groups. Figure 1A shows box plots of the distribution of maternal antibody reactivity with the four HIV-1 antigens at one serum dilution.

The anti-gp160 response was significantly higher in transmitting mothers ($P = .009$ and .005 for dilutions of 1/400 and 1/800, respectively). Anti-PID reactivity was also higher in transmitting mothers ($P = .055$ and .067 for dilutions of 1/400 and 1/800, respectively), while no association was found between anti-SUpep reactivity and transmission ($P = .322$ and

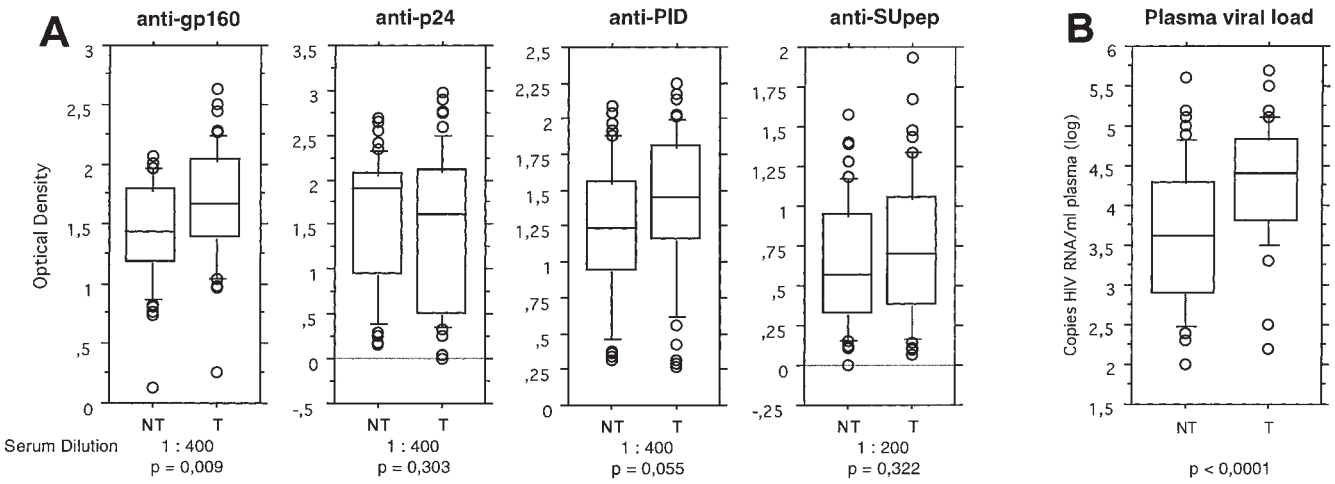

Figure 1. **A**, Box plots of distribution of maternal antibodies to HIV-1 antigens in transmitters (T) and nontransmitters (NT). Corrected optical density values obtained at 1 serum dilution are shown. Similar patterns of distributions were found at serum dilutions of 1:800 for gp160, p24, and PID (principal immunodominant domain), and 1:400 for SUpep (peptide from C-terminal sequence of extracellular gp120). Each box plot is composed of 5 horizontal lines representing 10th, 25th, 50th, and 90th percentiles of distribution of sera reactivity. **B**, Plasma HIV-1 RNA measured by quantitative reverse transcription–polymerase chain reaction. Box plot shows distribution of RNA copy number expressed as log-transformed values.

.220 for dilutions of 1/200 and 1/400, respectively). No significant difference in serum reactivity to p24 between transmitters and nontransmitters was observed ($P = .303$ and .500 for 1/400 and 1/800 dilutions, respectively). Considering that at the late stage of disease, there is a decline in the anti–HIV-1 immune response, we analyzed data after exclusion of mothers classified at CDC clinical stage C (3 nontransmitting and 2 transmitting mothers). The association between transmission and anti-gp160 reactivity still held ($P = .01$ and .007 for 1/400 and 1/800 dilutions), while the difference between transmitters' and nontransmitters' anti-PID reactivity became more significant ($P = .03$ for both 1/400 and 1/800 dilutions). The reactivities against HIV-1 antigens were lower in African than in Caucasian mothers ($P < .0001$ for gp160; $P = .0194$ for p24; $P = .0764$ for PID for a dilution of 1/400 and $P = .0004$ for SUpep for a dilution of 1/200). However, the differences between anti-gp160 antibody reactivity between transmitters and nontransmitters were similar in both groups (median, 1.762 vs. 1.561, $P = .070$, and median, 1.377 vs. 1.177, $P = .060$, for Caucasian mothers and African mothers, respectively, for a 1/400 dilution).

We then asked whether there was a relationship between maternal antibody response and progression of disease in infected children. On comparison of the antibody responses of 12 transmitting mothers whose children rapidly progressed to disease with that of the 37 transmitting mothers whose children did not develop a severe form of disease, no significant differences were observed (not shown).

Virus load. p24 antigenemia was detected in 3 of 49 nontransmitters and in 14 of 49 transmitters ($P < .0001$). Accordingly, plasma HIV-1 RNA levels, although dispersed over a wide range in both groups (figure 1B), were higher in mothers who transmitted the virus ($P < .0001$). Median RNA levels, expressed as logs, were 4.4 and 3.6 in transmitters and nontransmitters, respectively. There was no difference in plasma virus loads between Caucasian and African mothers ($P = .626$).

Logistic regression analysis. Univariate logistic regression confirmed the correlation between anti-gp160 reactivity and transmission and the trend to an association between anti-PID reactivity and transmission (table 1). Multivariate stepwise logistic regression analysis indicated that among the maternal variables considered, the anti-gp160 response and virus load were predictors of the risk of transmission. Consideration of these two variables allowed correct classification in 72.4% of the cases: 69.4% of uninfected infants and 75.5% of infected infants were correctly classified. For an increase of 1 optical density unit in anti-gp160 reactivity, the risk of transmission increased by a factor of 3.4, and for 1 log increase in virus load, the risk of transmission increased by a factor of 2.8. No interaction terms reached the threshold value of $P = .20$ (not shown). In particular, the interaction terms between maternal origin and anti-gp160 reactivity ($P = .29$) or virus load ($P = .43$) were not significant, meaning that the associations between the risk of transmission and anti-gp160 reactivity or virus load were not found different in magnitude irrespective of mothers' ethnic origin. Anti-PID reactivity and antigenemia were strongly correlated with anti-gp160 reactivity and plasma viremia, respectively, and did not emerge as independent risk factors in the model.

Discussion

In the current study, we analyzed the humoral response to HIV-1 and plasma HIV-1 RNA levels in infected mothers in

Table 1. Maternal factors associated with risk of vertical transmission of HIV-1.

Variable	No. of patients	Univariate analysis			Multivariate stepwise analysis		
		Odds ratio	95% CI	*P*	Odds ratio	95% CI	*P*
Anti-gp160	98	3.5	1.3–9.4	.0116	3.4	1.1–9.9	.0270
Anti-p24	98	0.8	0.5–1.3	.3290			
Anti-PID	98	2.1	0.9–4.8	.0736			
Anti-SUpep	98	1.7	0.7–4.5	.2543			
Log virus load	98	2.9	1.6–5.1	.0002	2.8	1.6–5.0	.0004
Origin				.1623			
Caucasian	74	1.0					
African	24	0.5	0.2–1.3				
CDC stage				.4640			
A	79	1.0					
B or C	12	2.2	0.6–7.8				
Unknown	7	0.8	0.2–3.9				
p24 antigen				.0072			
Negative	81	1.0					
Positive	17	6.1	1.6–23.0				
CD4 cell count				.6887			
⩽350	23	1.0					
351–500	14	0.9	0.2–3.5				
>500	25	0.6	0.2–1.9				
Unknown	36	1.1	0.4–3.3				

NOTE. Serum dilutions used are 1:800 for gp160, p24, and PID (principal immunodominant domain) and 1:400 for SUpep (peptide from C-terminal sequence of extracellular gp120). Logistic regression analysis using serum dilutions of 1:400 for gp160, p24, and PID and 1:200 for SUpep gave similar results. CI, confidence interval.

relation to mother-to-child transmission of HIV-1 and to disease progression in infected children. Anti-gp160 and anti-PID antibody responses were associated with transmission from mother to child, while the anti-p24 response was not significantly different in transmitter and nontransmitter groups. In agreement with previous reports [14], maternal virus burden was strongly associated with transmission of HIV-1. By multivariate analysis, the anti-gp160 response and plasma HIV-1 RNA levels were found to be independently associated with the risk of transmission.

To identify a protective antibody response, several studies have focused on the humoral response to single neutralizing domains, such as the V3 loop, or to the external glycoprotein of the viral envelope, the gp120, where most neutralizing epitopes have been mapped. However, these domains, involved in cell tropism and neutralization, are highly variable, and quantitative analysis of serum reactivity against peptides corresponding to these regions could be affected by sequence divergence of infecting virus and peptides used in the assay. By focusing on conserved immunogenic domains of the envelope, and by using ELISAs that permit detection of low-affinity cross-reactive antibodies, our goal was to study the anti-viral response, including nonneutralizing or enhancing antibodies, and thus contribute to more detailed dissection of the role of the humoral response in mother-to-child transmission of HIV.

We found that maternal serum reactivity to gp160 and PID was more elevated in transmitting than in nontransmitting mothers. Higher anti-PID reactivity for transmitting mothers, together with higher anti-V3 reactivity, was also observed in Congolese women [10]. Our data confirm and extend these observations in a cohort composed of mothers of Caucasian and African origins, in which the two ethnic groups displayed similar plasma virus loads. This suggests that the association of anti–HIV-1 envelope antibodies and mother-to-child transmission is of general significance and is not restricted to a particular African group. Interestingly, the significance of the association between anti-PID antibodies and transmission increased when the mothers with advanced disease (CDC stage C) were excluded from the analysis, suggesting that differences between transmitters and nontransmitters in antibody response against certain HIV epitopes may be lost owing to immunosuppression.

A high anti-gp160 response is a risk factor for mother-to-child transmission of HIV-1. Higher antibody production in transmitting mothers may be elicited by a higher load of replicating virus or, by favoring viral dissemination, may augment the virus load, or both phenomena may operate synergistically. Although it is difficult to differentiate between these possibilities, in the logistic regression model the anti-gp160 response was an independent variable that improved prediction based solely on plasma HIV-1 RNA levels. This observation argues for a direct link between these antibodies and perinatal transmission. However, the influence of other confounding factors, not considered in the present study, cannot be formally ex-

cluded. Viral sensitivity to antibody-mediated enhancement of infection has been advanced as a factor favoring mother-to-child transmission [15]. The conflicting data in the literature concerning the role of maternal antibodies in transmission could depend on the balance of opposed activities of antiviral antibodies, either neutralizing or enhancing.

In conclusion, our data, showing a correlation between the humoral response against HIV-1 envelope and perinatal transmission, suggest that antibodies directed to certain viral epitopes may be deleterious. This observation creates a caveat for immunotherapy in which anti-HIV antibodies are used for prevention of mother-to-child transmission and, more generally, should be considered in the design of anti-HIV vaccines based on envelope preparations.

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High Levels of Human Immunodeficiency Virus Type 1 in Blood and Semen of Seropositive Men in Sub-Saharan Africa

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High levels of human immunodeficiency virus type 1 (HIV-1) replication, as reflected in HIV-1 RNA concentrations in blood and semen, probably contribute to both rapid disease progression and enhanced sexual transmission. Semen and blood were collected from 49 Malawian and 61 US and Swiss (US/Swiss) HIV-1–seropositive men with similar CD4 cell counts and no urethritis or exposure to antiretroviral drugs. Median seminal plasma and blood plasma HIV-1 RNA concentrations were >3-fold ($P = .034$) and 5-fold ($P = .0003$) higher, respectively, in the Malawian men. Similar differences were observed in subsets of the Malawian and US/Swiss study groups matched individually for CD4 cell count ($P = .035$ and $P < .002$, respectively). These observations may help explain the high rates of HIV-1 sexual transmission and accelerated HIV-1 disease progression in sub-Saharan Africa.

Most human immunodeficiency virus type 1 (HIV-1)–infected people live in regions of Africa and Asia, where heterosexual transmission predominates [1]. The continued rapid expansion of this heterosexual epidemic likely reflects interactions among many behavioral and biologic factors. Analogous to other sexually transmitted diseases (STDs), the HIV-1 inoculum in semen is probably a major determinant of transmission from men. In support of this hypothesis, plasma HIV-1 RNA concentrations, which often predict semen levels [2, 3], correlate with transmission between stable partners [4]. Mucosal STDs significantly increase seminal plasma viral RNA [5], helping to explain their association with HIV transmission.

HIV-1 disease may progress faster in sub-Saharan Africa than in North America and western Europe [6]. Single plasma HIV-1 RNA measures independently predict prognosis in North American and European populations [7], but HIV-1 RNA concentrations in sub-Saharan Africans have not previously been reported. In this study, we examined the hypothesis that levels of HIV-1 in blood and semen in a Malawian population sampled in a cross-sectional fashion would exceed those in a comparable group of North American and European men, providing a biologic basis for the observed heterogeneity in rates of HIV-1 transmission and progression.

Materials and Methods

Study population. Forty-nine consecutive HIV-1–seropositive men attending dermatology clinics at Lilongwe Central Hospital (Malawian subjects) who served as a control group for a previously reported study [5] and 61 HIV-1–seropositive male volunteers from infectious diseases clinics at the University of North Carolina and the Kantonsspital, St. Gallen (US/Swiss subjects), were studied. The 61 US/Swiss men were an antiretroviral naive subset of a larger cohort of men studied [3]. Clinic evidence of mucosal or ulcerative STDs led to exclusion from the study. In Malawian men, urethral infection with *Neisseria gonorrhoeae, Trichomonas vaginalis,* or *Chlamydia trachomatis* was excluded by Gram's stain, culture, and *C. trachomatis* ligase chain reaction (Uriprobe LCR; Abbott Diagnostics, Abbott Park, IL) of urethral swabs. Malawian men were confirmed as HIV-1–seropositive by two EIAs (HIV-1/HIV-2 EIA; Genetic Systems, Seattle; Murex HIV-1 + 2, Murex Diagnostics, Dartford, UK), followed by Western blot (Organon Teknika, Durham, NC) for results that were equivocal. HIV-1 seropositivity was confirmed in all US/Swiss men by Western blot.

Specimen collection and laboratory procedures. Semen was processed as previously described [2]. US/Swiss samples were diluted in 2.5 mL of transport medium before processing. Blood plasma (BP) was separated and frozen at −70°C within 1 day of semen collection. HIV-1 RNA concentrations in BP and cell free

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Informed consent was obtained from all patients participating in this study. This research was conducted according to the human experimentation guidelines of the US Department of Health and Human Services. The protocol was approved by the University of North Carolina Committee on the Protection of Human Rights and The Malawi Health Sciences Research Committee.

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seminal plasma (SP) were quantified using nucleic acid sequence–based amplification assay (NASBA; Organon Teknika, Boxtel, The Netherlands) [2], with a sensitivity of 1000 RNA copies/mL.

T cell subsets were measured in blood collected from Malawian men at the same time as BP for HIV-1 RNA quantification. In the US/Swiss men, the CD4 cell count closest to the time of semen collection, within 1 month in each case, was used. HIV-1 subtype in Malawian men was determined by envelope V3 peptide immunoassay using antigens specific for clades A through F [8].

Statistics. Samples containing undetectable HIV-1 RNA were assigned a value half the assay detection limit (500 copies/mL). Arithmetic correction for dilution was made, except for undetectable RNA levels. The Mann-Whitney *U* test was used for comparisons between groups and Wilcoxon rank sum test for paired comparisons. For paired analysis, individual Malawian and US/Swiss subjects were matched for CD4 cell counts within 10 cells/μL of one another without knowledge of BP and SP HIV-1 RNA concentrations. Linear regression was used to identify independent predictors of BP and SP HIV-1 RNA.

Results

Characteristics of patient populations. Table 1 shows clinical and demographic characteristics of the subjects. In all but 5 cases, dermatologic disorders in Malawian men were minor, including superficial mycosis, seborrheic dermatitis, superficial bacterial infection, urticaria, pityriasis rosea, warts, molluscum contagiosum, eczema, psoriasis, and drug eruption. Five men had herpes zoster or primary varicella infection. No US/Swiss man described significant symptoms at the time of sample collection.

Comparisons of HIV-1 RNA concentrations in blood and semen. SP samples were collected from 49 Malawian and 61 US/Swiss men and BP from 42 and 49 men, respectively. Significantly higher levels of HIV-1 RNA were observed in Malawian than in US/Swiss BP and SP (median, 15.5×10^4 copies/mL vs. 2.88×10^4 copies/mL, $P = .0003$; and 1.51×10^4 copies/mL vs. 0.46×10^4 copies/mL, $P = .034$, respectively, figure 1A).

Table 1. Clinical and demographic characteristics of 49 Malawian and 61 US and Swiss (US/Swiss) HIV-1–seropositive men.

	Malawian	US/Swiss treatment-naive	*P**
Mean age (years) ± SD	32 ± 7.5	35 ± 8.0	.017
Median CD4 cell count (cells/μL), interquartile range	305 (190–479)	339 (160–500)	.73
Asymptomatic (CDC stage A)	36 (73%)	52 (85%)	.15
Symptomatic (CDC stage B or C)	13 (27%)	9 (15%)	
Active systemic infection	8 (16%)†	0	
Mode of HIV-1 acquisition	ND		
Heterosexual		14 (23%)	
Homosexual		37 (61%)	
Intravenous drug users		10 (16%)	

NOTE. In the Malawian group, 41 subjects had absolute CD4 cell counts available, and 42 had CD4 percentages available. In the US/Swiss group, all had CD4 cell counts done. ND, not determined.

* Continuous data were compared by Mann-Whitney *U* test, and dichotomous variables were compared by Fisher's exact test.

† Includes 4 subjects with dermatomal herpes zoster, 1 with disseminated varicella-zoster infection, 1 with secondary syphilis, 1 with active tuberculosis and possible drug-induced lupus, and 1 with possible tuberculosis (chronic cough, fever, night sweats, and weight loss.)

Paired analysis compared SP RNA concentrations in 33 pairs of Malawian and US/Swiss subjects individually matched for CD4 cell count (median CD4 cell counts, 299 cells/μL and 300/μL, respectively). BP HIV-1 RNA levels were available for 22 of these pairs. As shown in figure 1B, median Malawian SP HIV-1 RNA concentration was 1.70×10^4 copies/mL compared with 500 copies/mL (below detectable) in US/Swiss men ($P = .035$), with a median BP RNA of 15.5×10^4 copies/mL versus 2.34×10^4 copies/mL ($P < .003$).

Distributions of SP and BP HIV-1 RNA concentrations in the two study populations are illustrated in figures 1C and D. Fifty-seven percent (24/42) of Malawian men had blood HIV-1 RNA levels >125,000 copies/mL compared with only 20% (10/49) of US/Swiss men ($P = .0005$, figure 1D). The BP RNA level was below the assay detection limit of 1000 copies/mL in 12% of US/Swiss compared with 10% of Malawian men; SP RNA was undetectable in 42% and 22%, respectively. The differences between BP and SP log HIV-1 RNA values (equivalent to ratios between absolute BP and SP RNA levels) of individual subjects were similar in Malawian and US/Swiss groups (data not shown).

Malawian origin and CD4 cell count correlated significantly with the BP HIV-1 RNA concentration and independently predicted BP RNA level in multiple regression analysis (adjusted total $R^2 = .35$, $P < .0001$ for absolute CD4 cell count, $P = .004$ for country of origin). Although Malawian origin, CD4 cell count, BP RNA, and HIV-1 disease stage all individually correlated with SP HIV-1 RNA, the sole independent predictor following multiple linear regression analysis was BP RNA concentration (adjusted total $R^2 = .24$, $P = .0012$).

Eight Malawian men had evidence of active systemic infections at the time of sampling (see table 1 footnote), while all US/Swiss subjects were clinically well. The actively infected men had marginally higher BP HIV-1 RNA levels than the remainder of the Malawian group (median, 3.24×10^5 copies/mL [$n = 7$] compared with 1.41×10^5 copies/mL, $P = .39$) and similar SP RNA concentrations (median, 1.35×10^4 vs. 1.51×10^4 copies/mL, $P = .98$). When these 8 men were excluded from analysis, both BP and SP HIV-1 RNA concentrations remained significantly higher in Malawian than US/Swiss men ($P < .002$ and $P = .04$, respectively). The same observation held for paired analysis with matching for CD4 cell count, when the five pairs including an actively infected Malawian patient were excluded ($P = .008$ for comparison of BP HIV-1 RNA concentrations and $P = .006$ for SP RNA).

Of 42 Malawian men whose samples were submitted for subtype analysis, 40 had typeable virus. Thirty-seven had antibody directed only to peptide antigen specific for clade C, 1 had

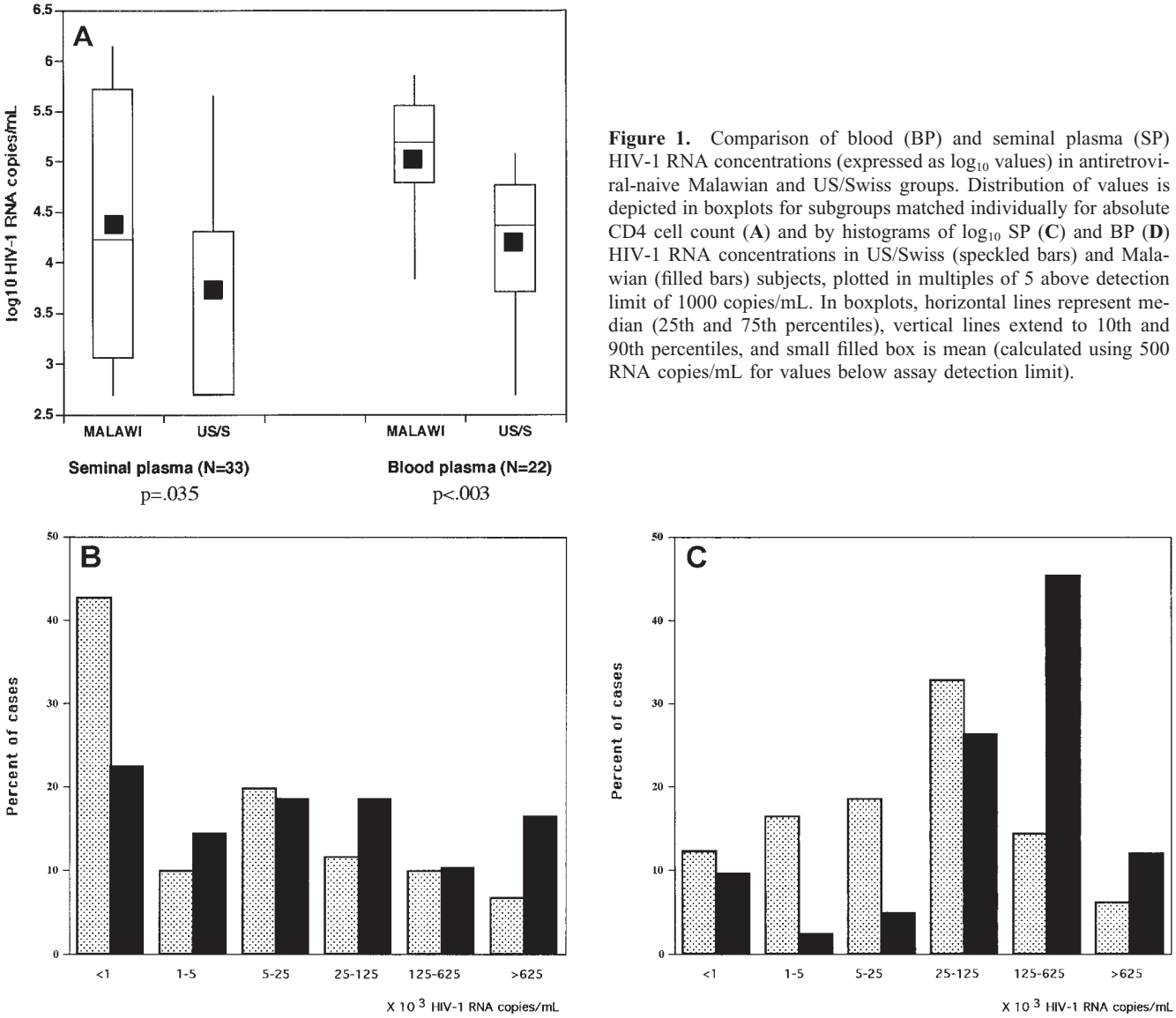

Figure 1. Comparison of blood (BP) and seminal plasma (SP) HIV-1 RNA concentrations (expressed as $\log_{10}$ values) in antiretroviral-naive Malawian and US/Swiss groups. Distribution of values is depicted in boxplots for subgroups matched individually for absolute CD4 cell count (**A**) and by histograms of $\log_{10}$ SP (**C**) and BP (**D**) HIV-1 RNA concentrations in US/Swiss (speckled bars) and Malawian (filled bars) subjects, plotted in multiples of 5 above detection limit of 1000 copies/mL. In boxplots, horizontal lines represent median (25th and 75th percentiles), vertical lines extend to 10th and 90th percentiles, and small filled box is mean (calculated using 500 RNA copies/mL for values below assay detection limit).

antibodies cross-reactive to clades A and C, 1 had antibodies to clades A, C, and F, and 1 had antibody to clade A only. All US/Swiss men had acquired HIV-1 infection in the United States or Switzerland and were considered likely to harbor clade B virus [1].

Discussion

We observed a 5- to 7-fold higher median BP viral RNA concentration in HIV-1–infected Malawian men than in a group of untreated US and Swiss men with similar CD4 cell counts. Recent studies indicate that blood levels of viral RNA are powerful predictors of progression to AIDS and death independent of CD4 cell count and other markers and that reductions in plasma HIV-1 RNA after antiviral drug therapy correlate with improved prognosis [7]. We found Malawian origin and residence to be a highly significant predictor of the BP HIV-1 RNA concentration, independent of CD4 cell count. High levels of HIV-1 replication could account for more rapid disease progression in sub-Saharan Africa than in the United States and Europe.

High levels of viral shedding in semen may partly explain the continuing rapid heterosexual spread of HIV-1 in sub-Saharan Africa [1]. We found 3- to 4-fold higher concentrations of SP HIV-1 RNA in Malawian men than in CD4 cell level–matched US/Swiss subjects receiving no antiretroviral therapy. In both groups, HIV-1 RNA levels in blood correlated with those in SP, the BP concentration being the only independent predictor of seminal HIV-1 shedding. Blood-to-semen HIV-1 RNA ratios did not differ between the 2 groups, suggesting that similar

factors were responsible for increased levels of virus in both body fluids in the Malawian men. Whether production of genital tract HIV-1 occurs in a separate biologic compartment, as suggested by previous studies [9], cannot be determined by the data presented here.

A number of host- or virus-related factors could explain the differences observed between BP and SP HIV-1 RNA levels of the 2 groups. Immune activation by infection or vaccination can reversibly elevate BP viral RNA concentrations [10]. Although 8 Malawian subjects had evidence of active systemic infection, they did not account for the elevated blood and semen RNA levels in the group as a whole. Nevertheless, common endemic infections such as tuberculosis and parasitic diseases could enhance viral replication while remaining clinically inapparent. High levels of serum immune activation markers have been described in HIV-1–infected Africans [11]. Furthermore, HIV-1–infected African immigrants to the United Kingdom progress at similar rates as seropositive Caucasians [12], suggesting strong environmental influences on the natural history of the disease.

Host genetic factors also influence HIV-1 pathogenesis and transmission. For example, heterozygosity for the Δ32 CCR-5 gene polymorphism slows progression to AIDS and is associated with reduced levels of plasma viral RNA [13]. The occurrence of the polymorphic CCR-5 allele has not been described in populations without Caucasian ancestry. HLA-linked and other genetic immune polymorphisms may also contribute to various rates of virus replication and disease progression between different populations.

Various efficiencies of viral replication between divergent viral subtypes in lymphoid and genital tract cells could influence the natural history and transmission of HIV-1. Soto-Ramirez et al. [14] recently reported that HIV-1 subtype E infects and replicates in epithelial Langerhans' cells more efficiently in vitro than do clade B viruses. The fact that we did not observe disproportionately higher levels of HIV-1 in the genital tract compared with that in blood in African men argues against more efficient in vivo genital tract replication of specific viral subtypes.

There were a number of limitations inherent in our study design and methods. Unequal priming efficiencies of subtypes B and C with NASBA could produce artifactual differences in viral RNA concentrations, although NASBA appears to be equally sensitive for all M group HIV-1 subtypes except clades A and G [15]. Given that the assay primers were designed and optimized to quantify clade B, differing amplification efficiencies should underestimate clade C HIV-1 RNA and bias against increased levels in the Malawian group. The relatively high assay detection limit resulted in censoring of information <1000 HIV-1 RNA copies/mL, and this result occurred more commonly in US/Swiss men. Finally, the retrospective and cross-sectional nature of the study and the necessity of selecting a comparison group of antiretroviral drug–naive US/Swiss men, who may have had a relatively stable disease course, caution against overinterpretation of our results.

The causes of the expanding heterosexual HIV-1 epidemics in sub-Saharan Africa and southern Asia are not clear. Behavioral factors may provide a partial explanation. However, distinct biologic aspects of the virus, the host, and the environment may be important determinants of regional patterns of HIV-1 spread and disease progression. The high HIV-1 RNA concentrations in blood and semen in this African population probably reflect interactions among genetic, immunologic, environmental, and viral influences. Modifiable environmental factors, especially acute and chronic bacterial and parasitic infections, may result in immune activation and increased systemic and genital tract HIV RNA concentrations. Dissecting the relative importance of these influences should enable development of new public health initiatives to control this epidemic.

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Proinflammatory Cytokine and Human Immunodeficiency Virus RNA Levels during Early *Mycobacterium avium* Complex Bacteremia in Advanced AIDS

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The relationship between *Mycobacterium avium* complex (MAC) bacteremia and proinflammatory cytokine and human immunodeficiency virus type 1 (HIV-1) RNA levels in AIDS was investigated. During a prospective study, blood samples were drawn monthly for mycobacterial cultures. Sera were available at baseline and onset of MAC bacteremia from 20 cases and at corresponding times from 19 controls. Mean interleukin-6 (IL-6) levels were 154% greater at the time of MAC bacteremia in cases than in controls. The IL-6 levels correlated with body temperature, serum tumor necrosis factor (TNF-α) levels, and alkaline phosphatase levels ($P \leq .004$ for each). Although TNF-α levels tended to rise more in MAC patients than in controls, the difference was not significant. However, among both cases and controls, serum TNF-α levels rose significantly from baseline to the time of last sample, irrespective of MAC infection ($P = .015$). Bacteremia was not associated with increased serum HIV-1 RNA levels. Thus, early MAC bacteremia is associated with increases in serum IL-6 levels, while TNF-α levels rise over time during advanced AIDS.

Disseminated *Mycobacterium avium* complex (MAC) disease is common during advanced AIDS. Cellular expression of tumor necrosis factor-α (TNF-α) is induced by mycobacterial proteins in vitro [1] and during tuberculosis in human immunodeficiency virus (HIV)–infected persons [2]. In addition, immune activating events such as febrile illnesses and immunizations cause transient increases in plasma HIV RNA. Cytokines that promote HIV expression in vitro, such as TNF-α and interleukin-6 (IL-6), may enhance HIV replication [3]. Among HIV-infected patients, active viral replication drives progressive CD4 cell depletion [4]. Current treatment strategies focus on minimizing HIV replication. The present study investigated whether early MAC bacteremia was associated with changes in serum IL-6, TNF-α, and HIV RNA levels in patients with AIDS.

Materials and Methods

Patients. Patients previously enrolled in a prospective clinical trial of clarithromycin prophylaxis in AIDS were studied [5]. Patients with <100 $CD4^+$ cells/mm^3 and baseline blood cultures negative for MAC were randomized to twice daily clarithromycin or placebo. Only patients from the placebo arm were included in the present analysis. Blood was drawn monthly for MAC surveillance cultures, and sera were sampled every 16 weeks. All patients with adequate stored sera and for whom matched controls could be identified were included. We identified 20 patients who developed MAC bacteremia (cases). A control was selected for each (except 1 patient who was a control for 2 cases). Cases and controls were

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matched for baseline $CD4^+$ cell count and time from baseline to last serum sample.

Antiretroviral use. Many patients received antiretroviral agents (zidovudine, didanosine, zalcitabine, stavudine, or foscarnet). These drugs were included in the analysis only if prescribed for at least 1 week before the pertinent serum sample. Antiretrovirals started after baseline but stopped at least 1 week before the pertinent sampling were not included in the analysis.

Sample processing. Whole blood was collected, and serum was separated by centrifugation and stored at −70°C. Sera from some time points were not available, and plasma samples were unavailable.

Cytokine assays. Sera were analyzed for TNF-α (Medgenix, Stillwater, MN) and IL-6 (R&D Systems, Minneapolis) in a blinded manner. The lower limit of IL-6 detection was 0.15 pg/mL. Lower values were censored to 0.15 pg/mL. The lower limit of TNF-α detection was 16 pg/mL. All results exceeded this level.

HIV RNA analysis. Sera were assayed for HIV RNA in a blinded manner using the branched-chain DNA method (Chiron) [6].

Mycobacterial cultures. MAC was isolated from blood using 7H11 agar or 7H12 broth (or both) by a radiometric method.

Statistical analysis. Nominal and ordinal variables were compared by χ^2, and interval and ratio variables were assessed by Student's *t* test or Pearson's correlation coefficient. For multiple linear regression, entry and exit probabilities of .05 and .10 were used, respectively. All *P* values were based on two-tailed tests. Subjects with missing data were excluded from individual analyses.

Results

Between November 1992 and July 1993, 682 patients with blood cultures negative for mycobacteria at baseline participated in a study of clarithromycin prophylaxis for disseminated MAC infection [5]. Of 334 randomized to placebo, 53 developed disseminated MAC infection, as defined by a positive blood culture. Twenty of these 53 patients, for whom adequate serum samples were available, were included in these analyses, as were 19 matched controls. None received specific MAC therapy during the study, including times when samples for the present analyses were collected. Of the 39 subjects, 90% were male, mean weight at baseline was 150 ± 5 lbs, age was 38.2 ± 1.1 years, $CD4^+$ cell number was 23.1 ± 3.6 cells/mm^3, $CD4^+$ cell percentage was 3.3% ± 0.5%, $CD8^+$ cell number was 530 ± 59 cells/mm^3, and $CD8^+$ cell percentage was 61.5% ± 2.0%. Groups did not differ with regard to sex or weight or CD4 or CD8 cell numbers or percentages ($P > .05$), although cases were slightly younger ($P = .046$).

For MAC cases, time from baseline to last serum sample (at or following onset of MAC bacteremia) ranged from 64 to 474 days (median, 250). Time from first positive MAC culture to last sample ranged from 0 (both obtained the same day) to 4 months. This interval was 0, 1, 2, 3, and 4 months for 6, 10, 1, 2, and 1 case(s), respectively. For controls, time between samples was similar, ranging from 19 to 484 days (median, 253).

Cases and controls had similar IL-6 and TNF-α levels at baseline (table 1). However, MAC bacteremia was associated with elevated IL-6 levels ($P = .03$). Similarly, when cases and controls were compared based on calculated differences between individual final and baseline IL-6 levels (to control for interpatient variability), the change in IL-6 levels remained significant ($P = .027$).

In contrast, although TNF-α levels tended to rise more among cases, the increase specifically associated with MAC bacteremia was not significant (table 1). Levels of TNF-α were higher among both groups at final sample time (42.5 ± 5.2 pg/mL) compared with baseline (28.4 ± 2.4 pg/mL), regardless of bacteremia ($P = .015$). There was no correlation between TNF-α levels and duration of storage (not shown).

The HIV RNA levels varied widely among the 39 subjects at all times, from <500 copies/mL to 4,266,000 copies/mL. Assays using serum have been shown to yield HIV RNA values 38% lower than plasma levels [7]. Mean baseline log HIV RNA level was similar in cases and controls (table 1), and HIV RNA levels at the time of bacteremia in cases did not differ from levels in control patients. To control for interpatient variability, the change over time in individual patients was calculated based on the first and last sample. This difference was not significant.

We further examined the relationship between clinical characteristics and MAC bacteremia. Other than a trend toward higher mean body temperatures (99.2 ± 0.4°F vs. 98.3 ± 0.2°F, $P = .059$) and lower hemoglobin levels (11.1 ± 0.6 vs. 12.4 ± 0.4 g/dL, $P = .069$) in cases versus controls, respectively, MAC bacteremia was not associated with changes in white blood, platelet, CD4, or CD8 cell counts or percentages, serum alkaline phosphatase, triglycerides, or body weight ($P > .05$).

Table 1. Comparison of serum levels of HIV RNA, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) in MAC cases and controls.

Characteristic	MAC cases	Controls	*P*
IL-6 level (pg/mL)			
Baseline sample	4.2 (0.6)	5.6 (2.3)	NS
Last sample*	13.7 (3.6)	5.4 (1.0)	.03
Last minus baseline	9.6 (3.6)	−0.2 (2.4)	.027
TNF-α level (pg/mL)			
Baseline sample	28.2 (3.9)	28.6 (3.0)	NS
Last sample	45.4 (7.8)	39.7 (6.5)	NS
Last minus baseline	17.8 (7.0)	11.5 (6.5)	NS
Log_{10} HIV RNA (copies/mL)			
Baseline sample	4.47 (0.16)	4.42 (0.17)	NS
Last sample	4.46 (0.12)	4.40 (0.20)	NS
Last minus baseline	−0.01 (0.14)	−0.02 (0.12)	NS

NOTE. MAC, *M. avium* complex infections. Mean values are shown (SE).
* Last sample = sample at or following onset of MAC bacteremia in cases and corresponding sample from controls.

In addition to baseline and time of MAC bacteremia, samples from intermediate time points were available from some patients, yielding a total of 53 sera from cases and 54 from controls. Analysis of these 107 samples (including baseline and final samples) revealed that serum IL-6 correlated with alkaline phosphatase ($r = .41$, $P < .001$) and body temperature ($r = .38$, $P < .001$). For TNF-α, weaker correlations were observed with triglycerides ($r = .25$, $P = .011$) and hemoglobin ($r = -.23$, $P = .022$). There was a strong correlation between IL-6 levels and TNF-α levels ($r = .38$, $P < .001$). Neither cytokine correlated with log HIV RNA.

We repeated the above analyses after controlling for antiretroviral use. At baseline, 19 (95%) cases and 17 (89.5%) controls were receiving at least one antiretroviral agent, for a mean of 1.10 ($\pm$0.10) and 1.16 ($\pm$0.14) agents per case and control patient, respectively. At the time of final sample, 5 patients in each group had started at least one new agent. In addition, some drugs received at baseline were discontinued, so that at study end controls and cases were receiving a mean of 1.0 $\pm$ 0.2 and 0.8 $\pm$ 0.1 agents, respectively.

When the 10 patients who started new agents were compared with the other 29 patients, there was no difference in IL-6, TNF-α, or log HIV RNA levels from baseline to final sample ($P > .05$ for each). Although the number of patients was small, among the 20 MAC case patients, there were no significant differences in changes in IL-6, TNF-α, or log HIV RNA levels among the 5 patients who had new antiretrovirals added compared with the 15 who did not.

Infectious or neoplastic complications other than MAC bacteremia could influence cytokine or HIV-1 RNA levels. However, such events were equally distributed between groups. At least one major infectious or neoplastic disease was diagnosed after collection of baseline but before last serum samples in 9 case and 9 control patients, and included cytomegalovirus retinitis (7), *Pneumocystis carinii* pneumonia (4), Kaposi's sarcoma (3), nonretinal cytomegalovirus disease (2), bacterial pneumonia (2), and lymphoma, pancreatitis, and intravenous catheter infection (1 each). All non-MAC infections except 2 were diagnosed at least 1 month prior to MAC bacteremia (1 in a case, 1 in a control). When these 2 subjects were excluded from analysis, final IL-6 levels remained higher in cases (14.4 $\pm$ 3.7 pg/mL) than controls (5.6 $\pm$ 1.0 pg/mL, $P = .031$), and final temperatures remained higher in cases (99.3 $\pm$ 0.4°F) than controls (98.3 $\pm$ 0.2°F, $P = .04$)

Discussion

This study demonstrates that early MAC bacteremia is associated with increased serum IL-6 but not TNF-α or HIV RNA levels. These findings were unexpected given the strong association between tuberculosis and increased expression of both proinflammatory cytokines and HIV [2, 8, 9]. In addition, a previous study demonstrated elevated TNF-α levels among patients with disseminated MAC infection [10]. Unlike the previous report, we identified patients from a prospective, randomized study which lasted for over a year [5]. Of interest, in this prospective study, serum TNF-α levels rose in all patients irrespective of MAC disease, suggesting that TNF-α expression increases over time in HIV disease as has been suggested by some [11] but not all [12] earlier cross-sectional studies.

The cytokines TNF-α and IL-6 participate in the host immune response to various intracellular pathogens, including mycobacteria. Increased production of TNF-α has been demonstrated during tuberculosis [1, 8], and serum TNF-α levels fall when HIV-infected adults with active tuberculosis receive effective therapy [13]. While the mechanisms involved are not well understood, exposure of peripheral blood mononuclear cells from healthy, tuberculin-positive volunteers to live *Mycobacterium tuberculosis* induces transcription of TNF-α and IL-6 mRNA. Similarly, mononuclear cells from patients with active pulmonary tuberculosis demonstrate elevated cytokine mRNA levels and increased production of TNF-α in response to exogenous stimuli [8]. During mycobacterial infection, TNF-α may be involved in immune pathways that result in fever and tissue necrosis, inhibit mycobacterial growth, and promote granuloma formation. Furthermore, TNF-α facilitates killing of intracellular MAC, while both TNF-α and IL-6 production may correlate with increased survival of MAC-infected macrophages [14].

The physiologic effects of TNF-α and IL-6 occur largely in the local tissue microenvironment at sites of infection. In the present study, activation of cytokine or HIV-1 expression may have been greater in the tissues during MAC bacteremia but was not reflected by circulating levels. Our findings of changes in IL-6 but not TNF-α or HIV-1 RNA levels may also reflect stage of MAC disease. In the present study, MAC bacteremia was probably diagnosed before extensive lymphoreticular organ involvement. This was predicted by the study design, which involved monthly blood cultures, regardless of symptoms, and is supported by the minimal clinical abnormalities specifically associated with bacteremia. This differs from the usual stage of diagnosis, by which time patients typically have high-grade fever and anemia. A report describing increased TNF-α levels during MAC involved such clinically overt disease [10].

In contrast to the present study, Havlir et al. [15] observed increased circulating HIV-1 RNA levels during early MAC bacteremia. Elevated IL-6 levels may precede HIV-1 RNA increases in bacteremic patients. It is possible that, in the present study, increased HIV-1 RNA levels would have been observed at later times.

Improved culture methods and newer macrolide antibiotics have influenced the approach to MAC infections in patients with AIDS [5]. We have shown that onset of MAC bacteremia may be associated with minimal clinical or laboratory evidence of disease, increased IL-6 levels, and little evidence of TNF-α or HIV RNA changes. The opportunity for early preemptive therapy at the time of minimal pathogenic effect should abort subsequent clinical disease and complications. However, in the

study by Pierce et al., surveillance blood cultures were performed monthly and patients were offered standard therapy when cultures became positive [5]. Despite this approach, mortality was greater among placebo recipients.

This study offered the opportunity to examine cytokine and HIV RNA levels during early MAC infection. If enhanced cytokine-induced HIV expression underlies the mortality seen in persons with progressive disseminated MAC infection, early prevention or treatment of MAC may prevent the cascade of events that accelerate the course of HIV-1 infection.

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Genotypic and Phenotypic Changes in the Emergence of *Escherichia coli* O157:H7

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***Escherichia coli* O157:H7 is a foodborne pathogen distinguished from typical *E. coli* by the production of Shiga toxins (Stx) and the inability to ferment sorbitol (SOR) and to express β-glucuronidase (GUD) activity. An allele-specific probe for the GUD gene *(uidA)* and multilocus enzyme electrophoresis were used to elucidate stages in the evolutionary emergence of *E. coli* O157:H7. A point mutation at +92 in *uidA* was found only in O157:H7 and its nonmotile relatives, including a SOR^+ $O157:H^-$ clone implicated in outbreaks of hemolytic-uremic syndrome in Germany. The results support a model in which O157:H7 evolved sequentially from an O55:H7 ancestor, first by acquiring the Stx2 gene and then by diverging into two branches; one became GUD^- SOR^-, resulting in the O157:H7 clone that spread worldwide, and the other lost motility, leading to the $O157:H^-$ clone that is an increasing public health problem in Europe.**

Escherichia coli serotype O157:H7 has emerged as a serious foodborne pathogen that has caused large-scale outbreaks of gastrointestinal illness in developed countries [1, 2]. These bacteria have several factors implicated in pathogenesis, including Shiga toxins (Stx) and a pathogenicity island called LEE that encodes proteins, such as intimin *(eaeA),* involved in attaching effacement [3, 4]. Evolutionarily, the O157:H7 serotype marks a distinct clone that is only distantly related to other Stx-producing enterohemorrhagic *E. coli* (EHEC) and is most closely related to an enteropathogenic *E. coli* (EPEC) clone of serotype O55:H7, a non–Stx-producing strain associated with infantile diarrhea [5].

The clonal nature of *E. coli* O157:H7 has facilitated its identification because these organisms, in contrast to most *E. coli* isolates, do not ferment sorbitol and lack β-glucuronidase (GUD) activity [2]. The gene encoding GUD *(uidA),* however, is intact and is nearly identical to the gene in *E. coli* K-12 with two nucleotide differences: an A→T mutation in the putative −10 promoter region and a T→G mutation at +92 in the structural gene [6]. The *uidA* +92 mutation is conserved among O157:H7 strains and can be detected by an allele-specific oligonucleotide probe [7].

Although O157:H7 is the predominant *E. coli* serotype incriminated in foodborne disease, various nonmotile and cytotoxigenic O157 variants have been isolated [8, 9]. In particular, Karch et al. [10] discovered a novel Stx-producing O157 strain that caused an outbreak of hemolytic-uremic syndrome (HUS) in Germany. In contrast to typical O157:H7 strains, these nonmotile O157 strains ferment sorbitol (SOR^+) and have DNA patterns distinct from those of typical O157:H7 isolates [10].

In the study reported here, we used multilocus enzyme electrophoresis to assess the clonal relationships among a variety of cytotoxigenic O157 strains, including nonmotile variants and other Stx-producing serotypes. We characterized strains for the presence of the *uidA* +92 allele, Stx genes, and sorbitol and GUD phenotypes. From a phylogenetic tree based on enzyme allele profiles, we formulated a model for the stepwise changes in virulence factors and phenotypic markers in the recent evolutionary emergence of *E. coli* O157:H7.

Materials and Methods

Bacterial strains. This study is based on analysis of 163 *E. coli* isolates, including 78 O157:H7 strains from contaminated foods and patients with hemorrhagic colitis or HUS, 4 Stx-producing $O157:H^-$ strains from HUS patients in Germany, 42 nonmotile O157 strains from the United States and Japan, 10 O157 strains of a variety of H types other than H7, and 33 strains of various O:H serotypes, 14 of which were Stx producers.

Strain characterization. All isolates were examined for the ability to ferment sorbitol and express GUD activity [2]. Shiga toxin genes *(stx1* and *stx2)* were detected by multiplex polymerase chain reaction (PCR) assays [11], and toxin production was confirmed serologically by use of Verotox-F (Denka Seiken, Tokyo).

To identify the +92 allele of the *uidA* gene, strains were examined by colony blots with an allele-specific probe (PF-27) as described previously [7].

Nucleotide sequence analysis. The 5′ terminus of the *uidA* gene was amplified by PCR with primers that anneal at −179 and

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+322 from the initiation codon [6]. The amplified 503-bp fragment was sequenced with the Silver Sequence kit (Promega, Madison, WI).

Clonal analysis. The genetic relationships among 46 representative strains were determined from allele profiles at 20 enzyme loci determined by multilocus enzyme electrophoresis [5]. Strains with identical profiles were assigned the same electrophoretic type (ET) and classified as members of a clone. Allele differences were used to estimate genetic distances between strains and to construct dendrograms by the average linkage algorithm [5].

Results

Clonal analysis and phenotypes. The multilocus enzyme genotypes of 46 strains resolved 15 ETs, whose genetic relatedness is shown in figure 1. The top cluster is the O157:H7 clone complex and comprises 5 closely related ETs that differ from one another by one or two enzyme alleles. All O157:H7 and most nonmotile O157 strains were ET1. Two O157 strains differed from this ET by alleles at a single locus; FDA 413 (ET2) had a fast-migrating peptidase and CDC G5101 (ET3) had a slow 6-phosphogluconate dehydrogenase electromorph. CDC G5101 was also unusual in that it was the only O157:H7 strain with a GUD^+ phenotype [9].

All of the O157:H7 were SOR^- and GUD^-, carried the *uidA* +92 allele, and had genes for Stx1, Stx2, or both. Serologic tests confirmed that the respective Stx was produced by those strains. Of the total of 42 nonmotile O157 strains examined, 41 carried the *uidA* +92 allele and had Stx genes, and except for 4 strains of ET4 (see below), all were SOR^- and GUD^-. Only 1 nonmotile O157 strain (USDA 7123, ET10) did not have the *uidA* +92 allele, and it was GUD^- and Stx^- (figure 1).

The O157:H7 complex also includes 7 O55:H7 isolates (ET5), which differ by a 6-phosphogluconate dehydrogenase allele from ET1 and are both SOR^+ and GUD^+ and, thus, phenotypically distinct from O157:H7 (figure 1). In addition, O55:H7 isolates are typically Stx-negative, although we found that 1 strain (USDA 5905) produced Stx2 (figure 1).

The most divergent member of the O157:H7 complex is ET4, comprising GUD^+ SOR^+ O157:H^- strains from Germany [12]. These isolates differed from ET1 by two enzyme alleles, a distinct malate dehydrogenase electromorph and a fast mannitol-1-phosphate dehydrogenase electromorph. These strains were positive for Stx2 and also carried the *uidA* +92 allele.

The bottom cluster of the dendrogram (ET6–ET15) is composed of non-H7 flagellar types and nonmotile O157 strains, as well as some representative Stx producers. All of these strains had ETs that differed by multiple alleles from the O157:H7 complex. None of the strains in the bottom half of the dendrogram had the *uidA* +92 allele.

Comparison of uidA gene sequences. To test further the genetic relationships in figure 1, we sequenced part of *uidA*

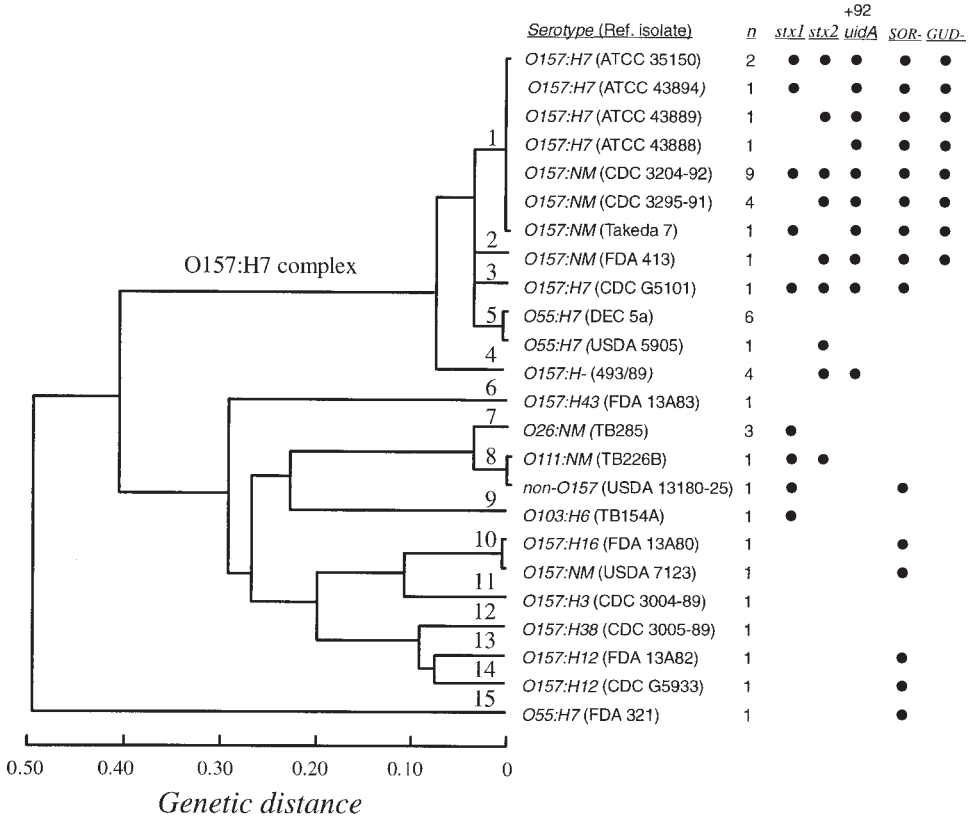

Figure 1. Dendrogram showing genetic distances among 46 representative isolates based on electrophoretic type (ET). ETs are numbered 1–15 and listed by serotype, with reference isolate given in parentheses (n = no. of isolates) and presence of specific genes (*stx1, stx2,* and *uidA* +92 allele) or phenotypes (SOR^- and GUD^-) indicated by solid circles.

from all 4 ET4 isolates and 4 of the 7 ET5 isolates and compared the sequences with that of O157:H7 [6]. The ET4 strains (O157:H$^-$) were identical to O157:H7, whereas the ET5 (O55:H7) strains had the −10 A→T mutation but did not have the T→G +92 mutation in *uidA*.

Discussion

The clonal analysis based on multilocus enzyme electrophoresis shows that O157:H7 and Stx-producing nonmotile variants (O157:H$^-$) belong to a genetically distinct clone complex that includes O55:H7 strains. The O157:H7 complex comprises a variety of phenotypic variants that appear to have recently gained and lost motility, Shiga toxin genes, and metabolic traits, such as the ability to ferment sorbitol. The close relationship of these strains is supported by the finding that a point mutation at +92 in *uidA* occurs in O157:H7 and its nonmotile relatives, including an atypical O157:H$^-$ clone implicated in outbreaks of HUS in Germany [10, 12] and in central Europe [13]. Comparative sequencing confirmed that these nonmotile O157 strains carried the same −10 and +92 mutations in the *uidA* as did O157:H7. Interestingly, ET5 (O55:H7) had the −10 but not the +92 mutation, suggesting that the O55:H7 *uidA* allele is an intermediate stage between the wild type allele and the *uidA* allele of O157:H7.

To reconcile the ET profile data, the variety of SOR and GUD phenotypes, and the distribution of the *uidA* mutations, we formulated an evolutionary model that posits a series of steps that have occurred in the emergence of the *E. coli* O157:H7. The model is based on three assumptions: that during divergence, the probability of loss of function greatly exceeds gain of function for metabolic genes; that the gain of function usually occurs via lateral transfer of genes; and that the sequence of events invoking the fewest total is the preferred model.

The evolutionary steps outlined in the model (figure 2) begin at the left with the ancestral or primitive states and progress to the right to the contemporary or derived states. The model begins with an EPEC-like ancestor that we assume, like most present-day *E. coli,* to be able to express β-glucuronidase (GUD$^+$) and ferment sorbitol (SOR$^+$). From this EPEC-like

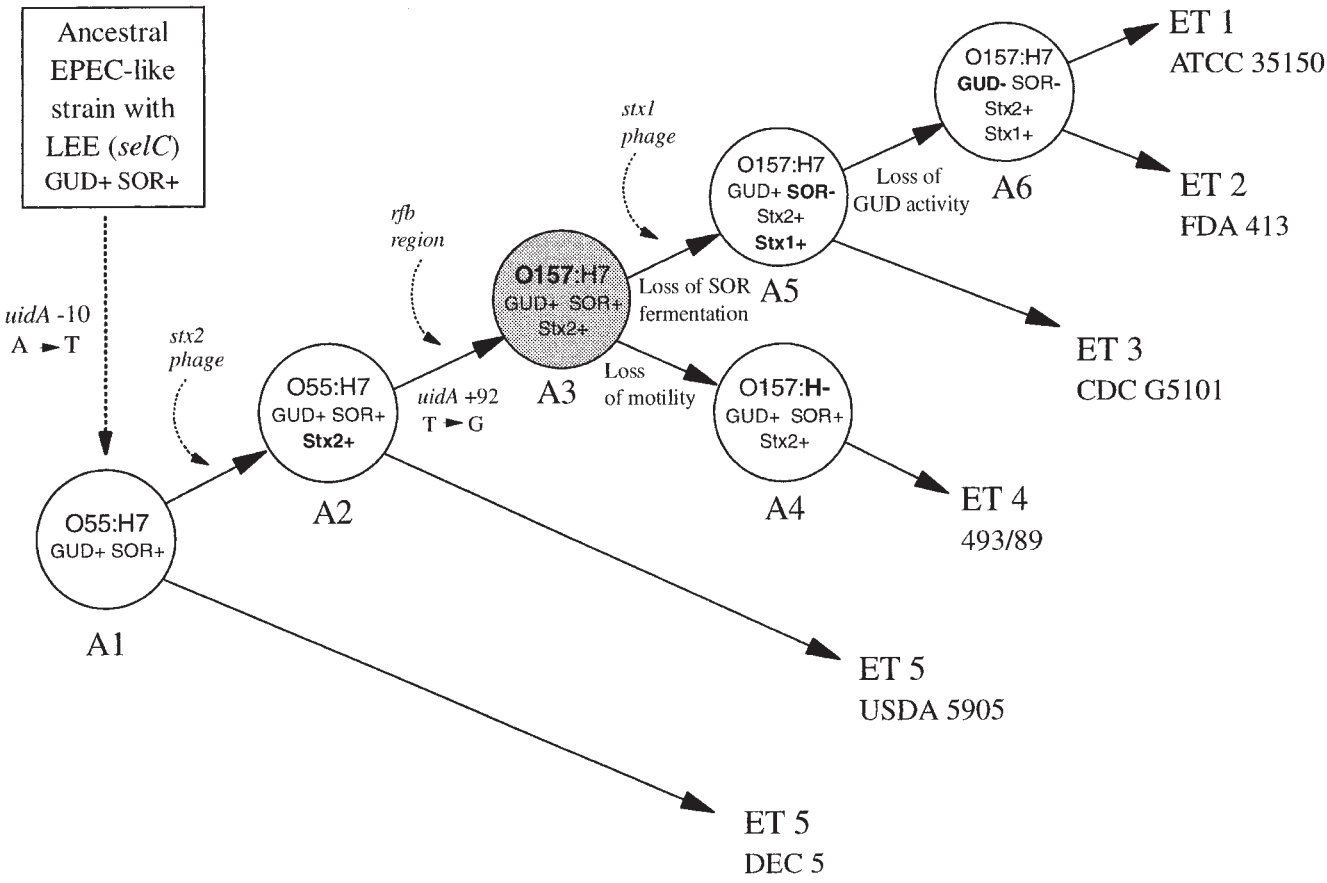

Figure 2. Proposed evolutionary model for emergence of O157:H7 complex based on mutations in *uidA,* Stx production, SOR and GUD phenotypes, and multilocus enzyme electrophoretic profiles of *E. coli* O157:H7 and its relatives. Phenotypes of ancestors A1–A6 are shown; changes predicted to have occurred are in bold. Representative isolates are given below each electrophoretic type (ET). Strain with tracts of ancestor A3 (shaded circle) has not been reported.

ancestor, the immediate ancestor with the O55 somatic and the H7 flagellar antigens evolved. This ancestral cell, labeled A1 in figure 2, represents the most recent common ancestor of the ET5 (O55:H7) clone and O157:H7 and its relatives and is assumed to have the *uidA* −10 mutation that occurs in the entire O157:H7 complex. The next step, A1→A2, was the acquisition of *stx2,* presumably by transduction by a toxin-converting bacteriophage, resulting in an Stx2-producing O55:H7 strain. This primitive stage is represented by strain USDA 5905 in ET5 (figure 1). From A2→A3, the *uidA* +92 mutation occurred and the somatic antigen changed from O55 to O157 to give rise to a hypothetical O157:H7 Stx2-producing ancestor (A3) that retained the GUD^+ SOR^+ phenotype. Thus far, we are not aware of an actual strain that has been isolated with this combination of traits. Although it is not shown in the model, we also postulate that A3 acquired the EHEC plasmid [14], because the plasmid is present in the descendants of this ancestral cell.

The findings of Bilge et al. [15] suggest that the transition from O55 to O157 antigen occurred as result of a lateral transfer of an *rfbE*-like region with homology to perosamine synthetase of *Vibrio cholerae.* The observation that the *rfbE*-like region is present in O157:H7 and genetically unrelated O157 non-H7 strains, but not in O55:H7, supports the hypothesis that a lateral transfer of this region occurred at this crucial A2→A3 stage in the emergence of O157:H7 from an O55:H7-like ancestor.

From A3, the model proposes that two distinct lines evolved. In the lower path, the lineage lost motility, to yield ancestor A4, which retained the Stx2 and the GUD^+ SOR^+ primitive phenotypes. During this divergence from A3, the lineage accumulated two enzyme allele mutations to give rise to the German $O157:H^-$ clone represented by ET4 (figure 1). Along the upper path, the lineage lost the ability to ferment sorbitol and acquired the Stx1 gene (presumably by phage conversion) to give rise to an intermediate A5 ancestral stage. The A5 has the primitive traits, GUD^+ and $Stx2^+$, and has the derived states (SOR^-, $Stx1^+$) in the combination of traits seen in strain CDC G5101 (ET3). In the model, A5 then lost GUD activity, producing the immediate ancestor (A6) of the common O157:H7 clone (ET1), which spread globally, and a rare variant (ET2), which differs by a single enzyme allele from ET1. Recent loss of Stx genes and motility in nature, or during isolation and culture, would account for the variants among isolates of the ET1 clone (figure 1).

In summary, the phylogenetic model diagrammed in figure 2 makes specific predictions about the history of descent and the order of acquisition of virulence factors in the emergence of the virulent O157:H7 serotype. The model predicts that both O157:H7 (ET1) and the German $O157:H^-$ (ET4) were derived from an EPEC-like O55:H7 ancestor, which carried *eaeA* and acquired the Stx2 gene. This proposition is supported by the similarities between these strains and by the presence of identical mutations in *uidA*. The German $O157:H^-$ clone, however, represents an early diverging member of the O157:H7 clone complex, which retained the ancestral ability to ferment sorbitol and to express β-glucuronidase activity. The model also stipulates that *stx2* was acquired once and at an early stage, before the somatic antigen transition to O157 and prior to the acquisition of the EHEC plasmid and *stx1*. Hence, aside from the *eaeA* and the LEE region, which already existed in the ancestor, the model predicts that Stx2 gene has been evolving in the O157:H7 genome for a longer time than other virulence factors, such as *stx1* and genes on the EHEC plasmid.

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Serogroup B, Electrophoretic Type 15 *Neisseria meningitidis* in Canada

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Invasive meningococcal disease is nationally reportable in Canada. In recent years, a serogroup C genotype, designated electrophoretic type 15 (ET15), has been the most frequently isolated meningococcal genotype in Canada and has caused epidemics across the country. Between August 1993 and September 1995, there were 9 cases of invasive meningococcal disease caused by a variant of this genotype, expressing group B capsular polysaccharide. The appearance of serogroup B:ET15 was related temporally and geographically to mass immunization campaigns designed to control serogroup C meningococcal disease in Canada. Since there is no vaccine available to control serogroup B meningococcal disease, the appearance of this variant may have public-health significance if it demonstrates the same epidemic potential as its serogroup C counterpart.

In Canada, invasive meningococcal disease (IMD) has been a major public-health issue during the last decade. From 1985 to 1993, there was an annual increase in the national incidence of IMD that was associated with the emergence of a new serogroup C clone. Multilocus enzyme electrophoresis placed this clone in the electrophoretic type (ET) 37 complex [1] but distinguished it by identification of a unique allelic variant for the enzyme fumarase [2]. Isolates belonging to this clone, designated ET15, were predominantly serotype 2a, subtypes P1.2,5. From 1988 to 1993, the percentage of all laboratory-confirmed invasive meningococcal disease in Canada attributable to serogroup C:ET15 increased from 2.0% to 51.8%. Epidemiologically, ET15 was associated with a significant increase in IMD incidence among persons 5–19 years old and a significant increase in the case fatality rate for all ages [3]. In response to this increase in serogroup C disease, there were 9 mass immunization campaigns (defined as >20,000 people immunized in a campaign) against meningococcal disease in Canada between 1992 and 1994. Serogroup C:ET15 has also been increasingly responsible for epidemic and endemic disease in the United States [4], England and Wales [5], and the Czech Republic [6].

In contrast to serogroup C:ET15, which has caused several community and institutional outbreaks [7], serogroup B *Neisseria meningitidis* has been responsible primarily for endemic disease in Canada. From 1987 to the present, most typeable serogroup B meningococcal isolates have been serotype 15 or 4 [8]. Here, we describe the epidemiologic and microbiologic characteristics of 9 cases of invasive serogroup B, serotype 2a, ET15 *N. meningitidis* found in Canada since 1993. This genotype may be especially interesting to public-health officials, because of its emergence following mass immunization to control serogroup C:ET15 disease, its potential for epidemic disease (given the behavior of its serogroup C counterpart), and the lack of an effective vaccine for serogroup B *N. meningitidis.*

Methods

In Canada, IMD is nationally notifiable according to standardized case definitions [9]. The Laboratory Centre for Disease Control (LCDC) maintains a national database that correlates case-by-case epidemiologic reports with results from laboratory evaluation. The epidemiologic reports include the date of disease onset and the patient's age, sex, residence (health unit), clinical syndrome, and outcome.

All sterile-site meningococcal isolates are forwarded to the LCDC for serogrouping, serotyping, and serosubtyping at the National Laboratory for Bacterial Diseases [10]. Multilocus enzyme electrophoresis and pulsed-field gel electrophoresis, as described previously [2, 7], are used routinely to characterize all serogroup C meningococcal isolates as well as serogroup B isolates that are suspected of belonging to the ET37 complex.

Results

From 1985 to the present, there have been 9 cases of invasive infection with serogroup B:ET15 *N. meningitidis* in Canada (table 1). All cases occurred between August 1993 and September 1995. Eight (89%) of the isolates came from the provinces of Québec (6 isolates) and Saskatchewan (2 isolates). None of the cases were epidemiologically linked. Two of the isolates were from patients in the 5–19 years age group. Three isolates were recovered from blood and 5 from cerebrospinal fluid. There was one fatality, a 3-month-old child. Two patients in Québec (cases 5 and 6) had been previously immunized with an A/C bivalent meningococcal vaccine as part of a mass immunization campaign. In cases 3 and 9, the patients had never been immunized. The immunization status of the remaining 5

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Table 1. Cases of invasive disease caused by serogroup B, electrophoretic type 15 *N. meningitidis* in Canada.

Case no.	Province	Date of onset (d/m/y)	Age (years)	Sex	Vaccine status	Outcome	Site of isolate	Serogroup: serotype: subtype
1	Québec	24/8/93	20	M	U	Survived	CSF	B:2a:P1.–
2	Québec	13/9/93	48	F	U	Survived	CSF	B:2a:P1.2,5
3	Saskatchewan	2/11/93	3 m	M	N	Died	Blood	B:2a:P1.2,5
4	New Brunswick	22/4/94	5	F	U			B:2a:P1.–
5	Québec	14/12/94	4	M	Y	Survived	CSF	B:2a:P1.2,5
6	Québec	11/1/95	2	F	Y	Survived	CSF	B:2a:P1.2,5
7	Québec	15/2/95	56	F	U	Survived	Blood	B:2a:P1.2,5
8	Saskatchewan	23/2/95	19	F	U	Survived	Blood	B:2a:P1.2,5
9	Québec	30/9/95	2	M	N	Survived	CSF	B:2a:P1.2,5

NOTE. d = day, m = month, y = year, M = male, F = female, N = not immunized, Y = immunized, U = immunization status unknown, CSF = cerebrospinal fluid.

patients was unknown, although 2 would not have been eligible to receive publicly funded meningococcal vaccine as part of any immunization campaign because of age (case 3, 48 years, exceeding maximum age of 20 years) or location (case 4, from a population in a nonimmunized area of New Brunswick). The remaining 3 patients with unknown vaccine status would have been eligible for publicly funded vaccine because of their ages and locations of residence.

All of the B:ET15 isolates were serotype 2a. Seven were subtype P1.2,5 and 2 were nonsubtypeable (P1.–).

In 1993, 1994, and 1995 there were, respectively, 393, 361, and 305 cases of invasive meningococcal disease in Canada, corresponding to yearly incidences of 1.4, 1.3, and 1.0/100,000, respectively. Of all laboratory-confirmed cases during this period, 39% were caused by group B strains, of which 36.4% were nonserotypeable, 28.7% serotype 4, 16.8% serotype 15, 8.3% serotype 14, 3.4% serotype 2a, and 2.1% serotype 2b. Serogroup C strains accounted for 48.6% of all laboratory-confirmed cases during the same time period. Among these, 87.9% were serotype 2a, 8.9% were nonserotypeable, and 3.2% were other serotypes, including 14, 15, 4, and 2b. Between 1993 and 1995, ET15 accounted for 93% of all serogroup C isolates in Canada.

Discussion

This report describes the appearance, in Canada, of a variant form of a prevalent invasive serogroup C meningococcal genotype (C:2a:P1.2,5:ET15), that instead expresses serogroup B capsular polysaccharide.

Meningococcal strains that are genetically similar to each other but express serogroup B or C capsular polysaccharide antigens have also been described in countries other than Canada [1, 11, 12]. The repeated observation of such variants at different times and places and in different chromosomal backgrounds suggests that they arise frequently in the bacterial population, as meningococcal strains carrying genes specifying different capsular polysaccharides co-colonize the human nasopharynx. The rate at which this occurs has yet to be estimated. In the US Pacific Northwest, capsular switching from serogroup B to C was described among a group of ET5-complex isolates during a 1994–1995 outbreak. Molecular genetic analysis of these isolates suggested that the capsule-switching phenomenon resulted from genetic transformation and recombination at a locus within the capsule-biosynthetic *(syn)* operon [12]. The C-to-B capsular switch reported here is the reverse of this B-to-C switch, but analogous genetic events may have preceded the appearance of B:ET15 isolates in Canada.

Two general types of explanation could be advanced for the recent appearance of these B:ET15 strains. The first invokes a fitness advantage for B-encapsulated variants of a prevalent C-encapsulated genotype. This could occur if the B-encapsulated variants were subject to less intense selection pressure from the immune systems of the population of human hosts, because they carry a polysaccharide antigen that is both different from that of the more prevalent group C strains (to which many potential hosts would already possess antibodies) and less immunogenic [13]. The resulting fitness advantage of the B variants may be modest. However, because the background chromosomal genotypes (here represented by the 2a:P1.2,5:ET15 array of markers) of the two capsular variants would be extremely similar, one might expect the B and C forms to occupy very similar ecologic niches. Thus, one might reasonably expect that whatever factors have led to the recent rapid increase in prevalence of C:ET15 in Canada [3] might also apply to B:ET15, and perhaps that intergenotypic competition between B:ET15 and its C:ET15 counterpart contributed to the appearance of the former.

We also raise the question of whether the recent appearance of invasive disease due to serogroup B:ET15 strains in Canada may be related partly to the widespread use of meningococcal vaccine in response to epidemics of C:ET15 (figure 1). Elementary population genetics theory indicates that the probability of escape from random loss of a new, selectively advantageous

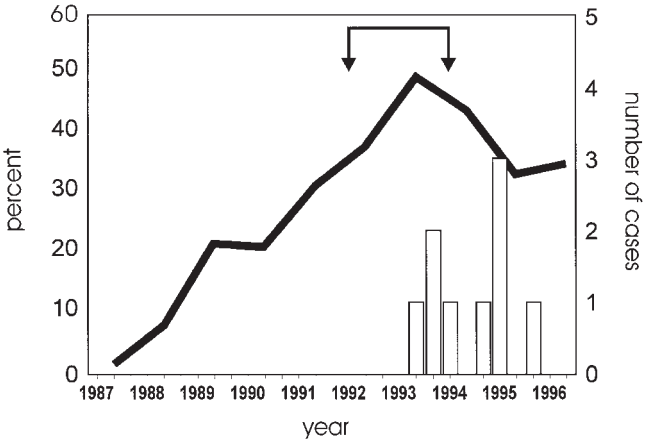

Figure 1. Percentage of all cases of laboratory confirmed meningococcal disease attributable to serogroup C, electrophoretic type 15 (ET15) in Canada between 1987 and 1996 is shown (solid line) in relation to appearance of cases of serogroup B:ET15 isolates (open bars). Period during which mass immunization campaigns occurred in Canada is bracketed by arrows.

genetic variant in a population, as well as its rate of frequency increase if it does escape such random loss, is directly proportional to the magnitude of its selective advantage [14]. Thus, one effect of mass vaccination might have been to intensify selection pressure past a critical threshold, such that the frequency of B:ET15 variants in the nasopharyngeal meningococcal population rose to a point where they began to appear in cases of IMD.

It is suggestive that 7 of 9 invasive B:ET15 isolates were identified after mass immunization in Québec and Saskatchewan, provinces that had carried out the largest campaigns. Québec immunized all persons 6 months to 19 years old (~1,613,000 people) between December 1991 and May 1993, and Saskatchewan immunized all persons 2–19 years old (~258,000 people) in November and December 1993. By providing a large segment of the population with antibody against serogroup C capsular polysaccharide, such immunization could have been another factor influencing the relative fitnesses of closely related strains of *N. meningitidis* carrying different capsular antigens. It is also noteworthy in this context that invasive B:ET15 strains have not yet been identified in the populous provinces of British Columbia or Ontario, which used smaller-scale mass immunization campaigns to control epidemic C:ET15. Though circumstances differed in each province prior to immunization, the latter two provinces targeted specific health units and communities, while Québec and Saskatchewan provided vaccine to large segments of entire provincial populations.

Another form of selection-based explanation is that vaccination might have influenced the representation of B:ET15 strains among those causing IMD, not because of direct competition between meningococcal genotypes in the nasopharyngeal environment but because of natural or vaccine-induced protective immunity to serogroup C invasive disease. It is important to point out, first, that at least 2 and likely 4 of the 9 patients in reported cases of B:ET15 disease did not receive vaccine (see Results). This would mean that vaccine-induced immunity of these individuals per se cannot be a direct contributing factor in their having contracted IMD due to B:ET15 meningococci. Second, although such protective immunity may indeed have contributed to the recent decline in the overall incidence of group C disease in Canada (figure 1), it is not clear how this factor alone could explain the recent appearance of the B:ET15 variants. If the latter had been present at significant frequencies in the meningococcal population before vaccination, one would expect them to have been observed at that time. However, it should also be clear that it is not possible at present to distinguish conclusively between explanations based on selection acting in the nasopharynx and those based on protective immunity to invasive disease.

Finally, despite the apparent plausibility of a selection- and/or competition-based explanation for the appearance of B:ET15 in Canada, alternative scenarios should also be considered. Foremost among these is the possibility that insufficient selective advantage of any kind accrues to B:ET15 variants to have caused their recent rise in frequency among cases of IMD, and that the latter effect is simply what one would expect, given a moderately high rate of transformation and recombination at the capsular-polysaccharide locus and high prevalence of a well-studied and closely observed genotype (C:ET15) on which selectively neutral B variants would be most likely eventually to occur and to be noticed. We plan to test this alternative explanation against the above-mentioned selection-based hypothesis, using a molecular population-genetic approach [12].

A switch from serogroup C:ET15 to serogroup B:ET15 may be especially significant from a public-health perspective, given the epidemic behavior of C:ET15 and because no vaccine exists against the B capsular polysaccharide. None of the B:ET15 isolates have occurred as part of an epidemic or cluster, and there have been no new cases of invasive B:ET15 infection since September 1995. This suggests that B:ET15 does not have the epidemic potential of C:ET15, at least in Canadian host populations at this time. However, comparisons of the epidemiologic behavior of serogroup B:ET15 and serogroup C:ET15 are limited by the small number of isolates, and continued surveillance will be necessary to document further this behavior in Canada and elsewhere, as well as to study the possible relationship with mass immunization. The potential for capsular switching may be one of the many factors public-health officials should consider when planning targeted or mass immunization for epidemic or endemic meningococcal disease.

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Vaccination with a *Haemophilus influenzae* Type b Conjugate Vaccine Reduces Oropharyngeal Carriage of *H. influenzae* Type b among Gambian Children

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The effect of a *Haemophilus influenzae* type b (Hib) polyribosylribitol phosphate–tetanus toxoid conjugate vaccine (Hib/PRP-T) on oropharyngeal carriage of Hib was studied during an efficacy trial in Gambian infants. Children were vaccinated with Hib/PRP-T and diphtheria-tetanus toxoids–pertussis (DTP) or DTP alone at ages 2, 3, and 4 months. Groups of 1000 children aged 1–2 years were studied each year for 4 years. Hib was detected by production of a halo on antiserum agar plates. Carriage was significantly lower among children fully vaccinated with Hib/PRP-T given with DTP (4.4%; 95% confidence interval [CI], 3.8%–5.7%) than among children fully vaccinated with DTP alone (11.0%; 95% CI, 8.9%–13.0%) (protective effect adjusted by year = 60%; 95% CI, 44%–72%; $P < .001$). Hib carriage varied by year among nonvaccinated children. Hib conjugate vaccines are likely to produce a herd protective effect in underdeveloped communities, as recorded in Europe and the United States.

Haemophilus influenzae type b (Hib) capsular polysaccharide vaccines protect against invasive disease in older children but not against oropharyngeal colonization with Hib [1]. In contrast, Hib capsular polysaccharide–protein conjugate vaccines reduce colonization by Hib, leading to a herd protective effect. Thus, Hib disease has virtually disappeared from many developed countries in which Hib conjugate vaccines have been introduced [2–7]. Takala et al. [3] were the first to report that vaccination with a Hib conjugate vaccine reduced colonization by Hib in Finish children. However, control rates for carriage were obtained before the vaccination study was started. Reductions in carriage of Hib among American Indian children and children in Atlanta have been reported following the introduction of a Hib vaccine [4, 5], but adequate control populations were not available for these studies.

The epidemiology of Hib disease differs significantly between developed and developing countries [8–10]. Hib carriage rates are often higher in developing than in industrialized countries, and colonization occurs at an earlier age and may be more intensive [9, 11–13]. Thus, it is uncertain whether Hib conjugate vaccines will be as effective at reducing carriage in developing countries as they are in industrialized communities.

In The Gambia, West Africa, Hib carriage in children is high and of short duration. A recently conducted efficacy trial of a Hib–tetanus toxoid protein conjugate vaccine (Hib/PRP-T) in Gambian infants has provided an opportunity for determining the effect of a conjugate vaccine on colonization by Hib in this developing country.

Subjects and Methods

The study was undertaken in the Western Region of The Gambia during the course of a Hib vaccine efficacy trial [14]. Vaccination coverage in The Gambia is high; >85% of children have received their third diphtheria-tetanus toxoids–pertussis (DTP) vaccine by the age of 1 year. The Gambian Hib vaccine trial was a double-blind, randomized, placebo controlled study; 21,490 infants received Hib/PRP-T with DTP and 21,358 received DTP alone at the ages of 2, 3, and 4 months. A booster dose was not given. Vaccines used for the trial were Hib/PRP-T (lot nos. S2801 and S3044), 10 μg/dose, and DTP (lot nos. G5801, J0102, and K5590; Pasteur Mérieux, Lyon, France). The outcome measures were protection against all invasive Hib disease, Hib pneumonia, and Hib carriage. The vaccine provided 95% protection against invasive Hib disease in children who had received three doses.

Study site. Children resident in the trial area received their vaccines at 20 main health centers or at vaccination clinics held in 43 smaller health centers. For the carriage study, two immunization centers, one in an urban and one in a rural area, were selected; there it was possible to recruit 20 children per week.

Study children. During 4 years, 500 well children were recruited sequentially each year when they presented to an immunization center. Recruitment was restricted to the months of August to December to minimize any possible effect of season on carriage. Similarly, recruitment was limited to children aged 1–2 years to restrict variability by age. The first 2000 children studied were recruited during the pretrial period or during the first year of the

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Consent was obtained from the family of each child who was admitted to the study. The trial was approved by the Gambia Government/MRC Ethical Committee.

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efficacy trial and had not received Hib/PRP-T vaccine. The final 2000 children were studied during the second and third years of the efficacy trial and had received either Hib/PRP-T vaccine given with DTP or DTP alone. In addition, all healthy contacts of cases of Hib disease identified during the trial who were ≤3 years old were studied to investigate the possible effects of vaccination in high-risk households.

Children were excluded if they had an illness requiring hospitalization or severe malnutrition or had received antibiotics during the preceding 2 weeks. A form was completed for each child, which recorded details of name, age, sex, date of birth, address, ethnic group, health card number, number of siblings, and Hib vaccination group. During the last year of the study, the number of siblings aged ≤3 years living in the household of each child was recorded also.

Sample collection. A swab was collected from the posterior pharynx with a sterile cotton-tipped swab (Sterilin, Stone, UK). The swab was plated directly onto Levinthal agar ASA plates containing burro anti-Hib antiserum (0.8 mL/10 mL; provided by J. B. Robbins, NIH, Bethesda, MD), bacitracin (5 U/mL), vancomycin (3.9 μg/mL), and clindamycin (0.78 μg/mL). Inoculated ASA plates were placed in a CO_2 (candle) jar in the field before transport to the laboratory. All swabs were collected by two investigators (R.A.A. and O.S.).

Bacteriology. In the laboratory, inoculated ASA plates were incubated at 35°C for 48 h at a 5% CO_2 concentration. Plates were examined for colonies surrounded by a halo, indicating formation of antigen-antiserum complexes. Plates were kept for another 24 h at 4°C before being considered Hib-negative. Control plates inoculated with a known Hib strain and a non–type b *H. influenzae* were included in each batch of assays. One or two colonies showing a halo were subcultured onto chocolate agar for X and V factor dependency determination and serotyped with Hib-specific antiserum (Murex Diagnostics, Dartford, UK). Of 426 strains of Hib isolated, 376 were biotyped and screened for antibiotic sensitivities by disk diffusion and tested for β-lactamase production by use of nitrocefin chromogenic cephalosporin (Unipath, Bedford, UK) [10]. MICs for 100 isolates were determined with E-test strips (AB Biodisk, Solna, Sweden). These included all 11 isolates that were resistant to one or more antibiotics by disk sensitivity testing and the first 22 or 23 isolates obtained each year.

Statistical analysis. Discrete data were compared between groups with the χ^2 test with Yates's correction or Fisher's exact test, as appropriate. Trend was investigated by use of the χ^2 test for trend. Continuous data were not normally distributed and were compared with a Wilcoxon test. Analysis of risk factors for carriage was done using unconditional logistic regression. The effect of age was investigated as a continuous and as a discrete variable with a cutoff of 18 months; the results were similar, but only the results of the latter are presented for ease of interpretation. Models were compared by change in deviance. The SAS System for Windows (SAS Institute, Cary, NC) was used for analyses.

Results

Study children. Four thousand children were swabbed; 14 were excluded because their demographic records could not be matched with the main trial database or because their age was incorrect, leaving 3986 children on whom the following analysis is based. Their median age was 16.5 months (range, 12.0–24.0), and the male to female ratio was 1:1. The mean number of siblings of carriers, ascertained from 445 children recruited in the third year of the trial, was 2.1 (range, 0–11); there was no significant difference in the number of siblings between carriers and noncarriers.

Hib carriage over time. The Hib carriage rate varied substantially with time among children not vaccinated with Hib vaccine and DTP. The prevalence of carriage was significantly lower in the pretrial year (8.2%) than during the first year of the trial (15.4%) ($\chi^2 = 24.4$; 1 *df*; $P < .001$) (table 1). It was 14.4% and 7.7%, respectively, among children fully vaccinated with DTP alone during the second and third years of the trial. Overall, 9.9% (95% confidence interval [CI], 8.0%–11.7%) of the children were carriers in the second year of the trial compared with 6.2% (95% CI, 4.7%–7.7%) in the third year of the trial ($P = .003$, Mantel-Haenszel χ^2 test stratified by vaccination status).

The households of 31 of 50 children with Hib disease were visited (the other 19 could not be traced), and 124 household contacts aged ≤3 years were studied. The numbers of contacts who were carriers were 13 (32.5%) of 40, 15 (23.8%) of 63, and 2 (9.5%) of 21 in the first, second, and third years of the trial, respectively (χ^2 test for trend = 3.82; 1 *df*; $P = .051$). Seven contacts had received Hib vaccine and DTP. If these children are excluded, the proportions of carriers among contacts were 13 (32.5%) of 40 in year 1, 15 (25.4%) of 59 in year 2, and 2 (11.1%) of 18 in year 3 (χ^2 test for trend = 2.8; 1 *df*; $P = .1$). A logistic regression model was constructed to take into account the influence of place of residence as well as the year of study. After adjusting for place of residence, there was evidence of a decline in carriage among contacts with time (change of deviance = 3.7; 1 *df*; $P = .05$).

Age, sex, and ethnic group. The prevalence of carriage was not influenced significantly by age, sex, or ethnic group.

Urban or rural differences. Equal numbers of children lived in rural or in urban areas (1989 and 1997, respectively). Overall, the proportion of carriers among children who lived in rural areas was 12.5% (95% CI, 11.1%–14.0%), compared with a prevalence of 7.4% (95% CI, 6.2%–8.5%) among children who lived in urban areas ($P < .001$). Carriage was higher in rural than in urban areas in each year of the study.

Effect of vaccination on carriage. Among the 1994 children who had received Hib vaccine and DTP or DTP alone, 1824 (91.5%) had received three doses, 123 (6.2%) two doses, and 47 (2.4%) one dose. Among children who had received three doses of vaccine, the median interval between the administration of the last dose of vaccine and the time when a swab was collected was 10.1 months (range, 0.16–19.8); this interval was similar in each year and for Hib vaccine plus DTP and DTP recipients. Of the fully vaccinated children who had received DTP alone, 11% (95% CI, 8.9%–13.0%) were carriers, compared with 4.4% (95% CI, 3.8%–5.7%) of those who had

Table 1. Hib carriage rates among children during 4 years of vaccine trial of Hib conjugate vaccine with or without diphtheria-tetanus toxoids–pertussis (DTP).

Year	No. of doses	DTP alone		Hib plus DTP	
		Urban	Rural	Urban	Rural
1	—	28/500 (5.6)	54/497 (10.9)	—	—
2	—	62/498 (12.5)	92/497 (18.5)	—	—
3	3	25/228 (11.0)	38/210 (18.1)	8/241 (3.3)	16/245 (6.5)
	2	0/11	4/13 (30.8)	4/13 (30.8)	1/18 (5.6)
	1	0	1/5 (20.0)	1/6 (16.7)	0/4
4	3	12/230 (5.2)	23/225 (10.2)	4/225 (1.8)	13/220 (5.9)
	2	1/10 (10.0)	3/24 (12.5)	0/13	1/21 (4.8)
	1	1/10 (10.0)	1/4 (25.0)	1/12 (8.3)	2/6 (33.3)

NOTE. Data are no./total (%).

received Hib vaccine plus DTP (Mantel-Haenszel χ^2 test stratified by year: protective effect = 60%; 95% CI, 44%–72%; $P < .001$). There was no significant difference in carriage rate between children partially vaccinated with Hib vaccine plus DTP or DTP alone, but numbers were small.

Multivariate analysis of risk factors for carriage. A logistic regression model showed that carriage rates were independently and significantly associated with Hib vaccination status, residence in a rural area, and year of study (table 2). A logistic regression model using only data from children who were in the efficacy trial gave similar results. In these analyses, after adjusting for year of study, sex, age, area of residence, and vaccination status, the time since the last dose of vaccine was not associated with the likelihood of being a carrier (change in deviance = .06; 1 *df*; $P > .8$).

Table 2. Multivariate logistic regression analyses showing independent effect of year of study, sex, age, area of residence, and vaccination status on risk of carriage.

Variable	Odds ratio (95% confidence interval)	*P*
Year		
1 (pre–efficacy trial year)	1.0 (0.7–1.4)	.8
2 (1st year of efficacy trial)	2.0 (1.4–2.7)	<.001
3 (2nd year of efficacy trial)	1.8 (1.2–2.5)	.001
4 (3rd year of efficacy trial)	1.0	
Sex		
Female	1.0 (0.8–1.2)	.8
Male	1.0	
Age		
<18 months	1.0 (0.8–1.2)	.9
⩾18 months	1.0	
Residence		
Rural	1.8 (1.5–2.3)	<.001
Urban	1.0	
Vaccination*		
Full	0.4 (0.2–0.5)	<.001
Partial	1.0 (0.5–1.9)	.9
Control	1.0	

* Fully vaccinated children received 3 doses of Hib vaccine plus diphtheria-tetanus toxoids–pertussis (DTP), partially vaccinated children received 1 or 2 doses of Hib plus DTP, and control children received 1–3 doses of DTP alone.

Characteristics of Hib isolates. Detailed bacteriologic analyses were done on 376 isolates; only 1 was β-lactamase–positive. This isolate was also resistant to ampicillin, chloramphenicol, and tetracycline (MICs of 4.0, 8.0, and 16.0 μg/mL, respectively). Tetracycline resistance was detected in 11 of 100 isolates for which MICs were determined (MICs of 12–32 μg/mL). Two isolates were multiply resistant; 1 was resistant to chloramphenicol, cotrimoxazole, and tetracycline (MICs of 8, >32, and 12 μg/mL, respectively) and the other was the β-lactamase–positive isolate. No strains were resistant to cefotaxime. The prevalent biotypes were I (92%), II (7.7%), and III (0.27%).

Discussion

We have found significant changes in the prevalence of Hib carriage in Gambian children over a 4-year period; the level of carriage peaked in the second year of the study without obvious reasons. The prevalence of carriage was higher in rural than in urban children in all years of the study. The reasons for this latter finding are not clear. Living conditions, including those associated with crowding, are little different in rural and urban areas of The Gambia. An analysis of other factors that might affect oropharyngeal carriage of Hib, such as the number of siblings in the household, showed no significant differences between rural and urban areas.

In many countries where Hib conjugate vaccines have been introduced, there has been a dramatic reduction in the incidence of Hib disease; this reduction was often greater than could be attributed to the direct effect of vaccination and included protection from disease in age groups not included in the vaccination program. This has been attributed to a herd protective effect achieved through a reduction in carriage [3–7]. Until

the present study, no information was available on the effect of Hib conjugate vaccines on carriage of Hib in developing countries. We have now shown that a PRP-T conjugate vaccine given during infancy reduced oropharyngeal carriage of Hib in fully vaccinated children by 60% during the second year of life in a community in which exposure to Hib is likely to be much more intense than in industrialized countries. It is also possible that the degree of colonization may have been reduced in immunized children, even when prevention of carriage had not occurred. The additional benefit of a herd protective effect following vaccination with a conjugate vaccine may boost the overall impact of a Hib vaccination program in developing countries, in which vaccine delivery system and coverage are often constrained.

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Humoral Immune Response to Tetanus-Diphtheria Vaccine Given during Extended Use of Chloroquine or Primaquine Malaria Chemoprophylaxis

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Immune suppression resulting from prolonged chemoprophylaxis and potential drug-vaccine interaction were investigated within the context of a randomized placebo-controlled trial that compared daily primaquine or weekly chloroquine administration for malaria prevention. After 11 months of prophylaxis, adult male subjects received a tetanus-diphtheria (Td) vaccination. Prophylaxis continued 4 weeks longer. Anti-tetanus and anti-diphtheria antibody levels were measured by ELISA at baseline and at 1, 3, 7, and 14 months after Td vaccination. All groups were comparable at baseline. Immunization triggered significant increases in anti-tetanus and anti-diphtheria IgG levels over each group's pre-Td baseline levels and those of an unvaccinated control group. Geometric mean anti-tetanus titers (GMTs) in the primaquine group were significantly higher than those of the placebo group at 1, 3, and 14 months. Anti-tetanus GMTs in placebo and chloroquine groups declined over 14 months to levels comparable to those of unvaccinated controls, but levels in the primaquine group remained significantly higher than in controls.

Recent trials in Indonesia and Africa have generated new interest in primaquine for malaria chemoprophylaxis [1–3]. In Indonesian men, a daily regimen of 0.5 mg of primaquine base per kilogram of body weight was well-tolerated and yielded protective efficacies against *Plasmodium falciparum* and *Plasmodium vivax* of >90% [3]. In this year-long trial, primaquine was associated with enhanced lymphoproliferative responses to mitogens and antigens [4]. This unanticipated result prompted an in vivo immunization trial designed to further reveal the immunomodulating effects of the drug or the malaria protection it conferred.

A tetanus-diphtheria (Td) vaccination was administered to groups treated with primaquine, chloroquine, or placebo in the last month of the trial. Subjects' in vitro lymphoproliferative responses to tetanus were measured at subsequent time points. Primaquine was associated with the highest initial post-Td responses of lymphocytes to tetanus, and no significant differences were observed between primaquine and placebo groups in the frequency, magnitude, or duration of these responses [5]. However, because protective immunity to tetanus is primarily if not wholly determined by levels of neutralizing antibody and because antimalarials can inhibit antibody response to rabies [6, 7], cholera, and typhoid vaccines [8], we were most interested in the drug's effects on humoral responses to the Td vaccination. This study compared anti-tetanus and anti-diphtheria IgG responses evoked by the Td vaccine among groups receiving primaquine, chloroquine, and placebo.

Materials and Methods

Subjects and prophylaxis. The chemoprophylaxis and Td immunization (5 limits of flocculation units [LfU] of tetanus toxoid and 2 LfU of diphtheria toxoid/0.5 mL intramuscularly) components of this study have been described [2, 5]. Only 2 of 72 subjects recalled a previous immunization (>10 years prior). None of the subjects had been in military service where there might have been greater likelihood of immunization. Primaquine (n = 30), chloroquine (n = 21), placebo (n = 21), and control without prophylaxis (n = 20) groups were comparable in terms of age (~30 years), weight (~50 kg), ethnicity (Javanese/Sundanese), and socioeconomic status (new transmigrant farmers). Primaquine-treated subjects had been malaria-free during the 12 months of prophylaxis, but an estimated 0.8–1.0 cases of malaria/person-year had occurred in the chloroquine and placebo groups. The monthly incidence of primary postprophylaxis malaria over the 3- and 7-month post-Td vaccine sampling points was comparable in the 3 groups (0.15–0.3 infections/person-month). Subjects were malaria-free at vaccination and were screened for infection at each sampling point. Those with symptoms and parasitemia were treated, and their samples were omitted from analysis because of potential malaria-induced immune suppression.

Anti-tetanus and anti-diphtheria IgG assays. Standardized ELISAs were used to blindly measure anti-tetanus and anti-diph-

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This work was conducted in accordance with US Navy and Republic of Indonesia regulations governing the protection of human subjects in medical research. American and Indonesian committees for the protection of human subjects reviewed and approved the procedures followed in this research. Written informed consent was obtained from each volunteer.

The views of the authors expressed herein do not purport to reflect those of the US Navy, the US Department of Defense, or the Indonesian Ministry of Health.

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The Journal of Infectious Diseases 1998;177:1762–5

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theria IgG levels in coded prophylaxis and control group sera that was collected at 5 time points: baseline (before Td vaccination) and 1, 3, 7, and 14 months after Td vaccination. Absorbance values for each sample were expressed as ELISA units (EU) and converted to international units (IU) by the formula: Sample EU/Reference EU × Reference Serum IU/mL. (The reference serum NCIV lot 1 contained 50 IU/mL anti-tetanus IgG and 2.9 IU/mL anti-diphtheria IgG). In accordance with current conservative recommendations, IgG titers ⩾0.1 IU/mL were considered protective [9].

Statistical analysis. Comparison between groups was based on log-transformation of each subject's IU/milliliter values and analysis of variance (ANOVA) or Kruskal-Wallace nonparametric test. Student's *t* test was used for paired comparisons when the ANOVA or Kruskal-Wallace test indicated significant differences among groups. Differences within or between groups in the proportion of subjects with protective IgG levels ⩾1.0 IU/mL were compared by χ^2 or Fisher's exact test. Two-tailed *P* values were calculated; the cutoff level for significance was $P \leqslant .05$.

Results

Baseline IgG responses. Baseline anti-tetanus IgG responses in primaquine, chloroquine, and placebo groups were comparable, with individual titers ranging from 0 to 0.44 IU/mL. Protective anti-tetanus titers were found in 47%, 52%, and 29% of the respective groups (figure 1, $P > .25$). Anti-tetanus geometric mean titers (GMTs) were 0.09, 0.09, and 0.07 IU/mL, respectively (ANOVA, $P = .66$). Individual baseline anti-diphtheria IgG titers ranged from 0.02 to 5.28 IU/mL. Protective anti-diphtheria titers were found in 93%, 95%, and 100% of the primaquine, chloroquine, and placebo groups, respectively. Anti-diphtheria GMTs were 0.49, 0.49, and 0.40, respectively (Kruskal-Wallace, $P = .66$).

Post-Td anti-tetanus IgG responses (figures 1, 2A). All but 1 of the 72 prophylaxis group subjects had increased anti-tetanus IgG titers 1 month after Td vaccination. The proportion of subjects with protective titers increased significantly ($P < .02$) in each group of vacinees. Conversion rates from unprotected to protected status in the primaquine (16/16) and placebo (15/15) groups were marginally greater ($P = .05$) than in the chloroquine group (7/10). Individual elevations in IgG ranged from 1 to >700 times those of baseline. There was no correlation between baseline levels and those attained 1 month after vaccination. Anti-tetanus IgG elevations 1 month after Td vaccination in primaquine, chloroquine, and placebo groups averaged 9.5, 5.6, and 5.2 times their respective baseline levels, and all group GMTs were significantly greater than at baseline ($P < .01$). Anti-tetanus GMTs 1 month after Td vaccination in the chloroquine and placebo groups were marginally higher than in the unvaccinated controls ($P < .04$). The anti-tetanus GMT in the primaquine group at this time was marginally above that of the vaccinated placebo group ($P = .04$) and significantly greater than that of the unvaccinated control group ($P < .001$).

In all vaccinated groups, the proportion of protective anti-tetanus titers and the GMTs fell from the peak levels attained 1 month post-Td. Relative to these peaks, end-point proportions with protective titers declined significantly in primaquine (100% vs. 82%, $P = .03$) and placebo (100% vs. 58%, $P = .001$) groups. End-point proportions of subjects with protective titers in the placebo (58%) and chloroquine (67%) groups fell

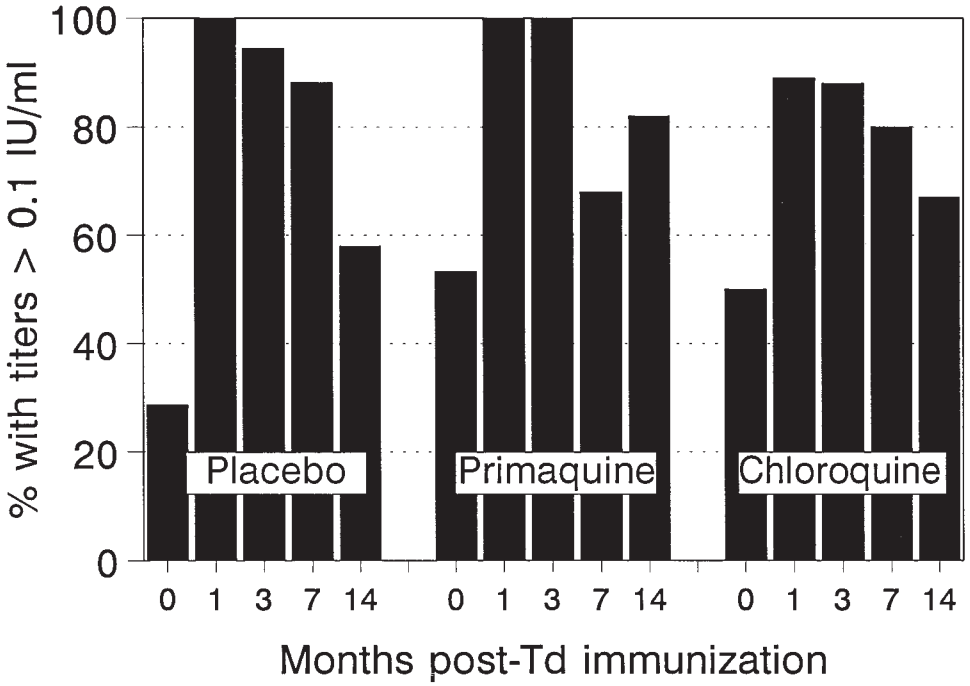

Figure 1. Proportion of subjects in prophylaxis groups with protective anti-tetanus IgG titers >0.1 IU/mL at baseline (0) and at 1, 3, 7, and 14 months after tetanus-diptheria (Td) immunization. Placebo ($n = 21$), primaquine ($n = 30$), and chloroquine ($n = 21$) groups were inoculated with Td at time 0.

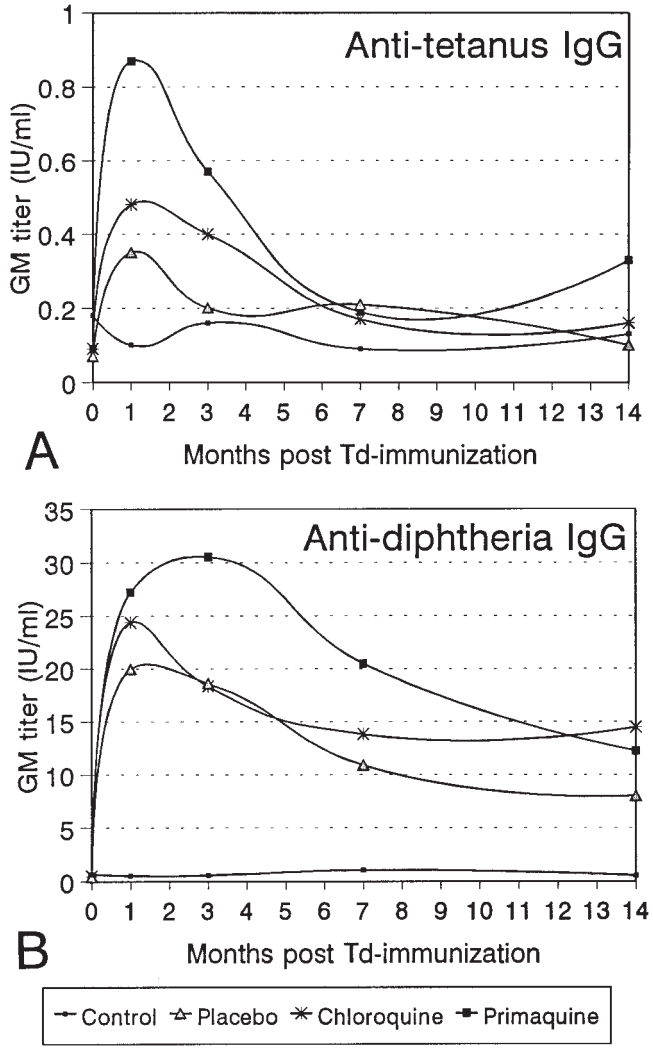

Figure 2. Geometric mean (GM) anti-tetanus (**A**) and anti-diphtheria (**B**) IgG titers (IU/mL) in control and prophylaxis groups at baseline (0) and at 1, 3, 7, and 14 months after tetanus-diptheria (Td) immunization. Placebo, primaquine, and chloroquine groups received Td inoculation at time 0; control group received neither prophylaxis nor Td. Sample size range by test group for time points: placebo (n = 16–21), primaquine (n = 22–30), chloroquine (n = 15–21), control (n = 8–13).

to levels comparable to their respective pre-Td vaccination baseline levels (placebo, P = .11; chloroquine, P = .50), but that of the primaquine group remained significantly above baseline (82% vs. 47%, P = .03). At end point, the proportion of primaquine subjects with protective titers was significantly greater than that of the unvaccinated control group (82% vs. 43%, P = .03), while proportions in placebo and chloroquine-treated groups were similar to that of the control (P > .27).

Anti-tetanus GMTs in the primaquine group exceeded those of the vaccinated placebo group at 3 (P = .03) and 14 months (P = .002) after Td vaccination. There was no statistical difference between GMTs of the placebo and chloroquine groups at any postvaccination sample point (P > .17). Despite falling significantly from the peak attained 1 month after Td vaccination, the end-point GMT of the primaquine group remained significantly higher than its baseline (P = .004) or that of the unvaccinated control group (P = .02). End-point GMTs for vaccinated placebo and chloroquine groups fell to levels comparable with their baselines (P > .10) and that of the control (P > .22). There was no significant difference between anti-tetanus GMTs calculated for the unvaccinated control group at any of the 5 time points (ANOVA, P = .42).

Post-Td vaccination anti-diphtheria IgG responses (figure 2B). Anti-diphtheria IgG titers 1 month after Td vaccination ranged from 1.7- to 257-fold greater than at baseline. Highest anti-diphtheria GMTs, measured 1 month after vaccination, were 27.23, 24.38, and 19.95 IU/mL for the primaquine, chloroquine, and placebo groups, respectively (ANOVA, P = .64), and averaged 58-, 62-, and 46-fold higher than their respective baseline GMTs. Anti-diphtheria GMTs of the primaquine, chloroquine, and placebo groups were comparable to one another (P > .26) but were significantly greater than their respective baseline GMTs (P < .0001) and those of unvaccinated controls (P < .0001) at each post-Td vaccination sample point. Anti-diphtheria GMTs declined over time in each vaccinated group. A significant decline from the peak 1 month after Td vaccination was measured in the chloroquine group 7 months after vaccination (P = .04) and in the primaquine and placebo groups 14 months after vaccination (P = .02 and .03, respectively). In all 3 vaccinated groups, subjects that attained the highest anti-diphtheria titers 1 month after vaccination generally also registered the highest end-point titers (r = 0.69–0.84). There was no correlation between anti-diphtheria and anti-tetanus antibody levels following immunization (r = −0.19 to −0.01). There was no significant difference between anti-diphtheria GMTs calculated for the unvaccinated control group at any of the 5 time points (ANOVA, P = .53).

Discussion

The results show that the attainment of protective anti-tetanus antibody titers and the magnitude of anti-tetanus GMTs were routinely highest in the vaccinated primaquine group. This finding complements the strong in vitro lymphocyte responses against tetanus that were measured in this treatment group [5]. Of interest, there was no correlation between cellular and humoral responses to tetanus: Men with the highest lymphoproliferative responses were equally likely to have either high or low anti-tetanus IgG titers. The heightened cellular and humoral responses observed in the primaquine group during the time of prophylaxis may relate more to the immunostimulatory properties of the drug than to its ability to prevent malaria-induced immune suppression. While it seems clear that long-term primaquine use was not immunosuppressive, the mechanisms underlying its effect on cellular and humoral immunity

are unclear and may relate to drug-induced leukocytosis or hematopoiesis [10].

Since no association has been shown between asymptomatic malaria infections and reduced primary or secondary responses to tetanus immunization [11], it is unlikely that undetected or subpatent malaria infections could account for the large differences in cellular and humoral response that we measured. Unrecalled previous tetanus immunizations in our subjects and their chance clustering may have accounted for the significant differences observed; however, baseline pre-Td responses, both humoral and cellular, gave no indication of such bias. Furthermore, rapidly declining post-Td anti-tetanus titers in even the highest responders, asynchrony between the magnitude of individual anti-tetanus and anti-diphtheria titers, and the absence of a correlation between subject age and titer gave no supporting evidence of prior immunization.

On the basis of chloroquine's established immunosuppressive quality [6–8, 12] and our observation of lymphoproliferative responses in the chloroquine group consistently below those of the primaquine and placebo groups [5], we had conjectured that anti-tetanus IgG levels would be similarly low. However, IgG titers in the chloroquine group exceeded or were comparable to those of the placebo group, and there was no difference at any sampling point in the proportion of subjects mounting protective titers. Nonintervention and possible enhancement of anti-toxin and anti-bacterial antibody responses have been previously reported for vaccinated African children maintained malaria-free by long-term chloroquine prophylaxis [11, 13].

The baseline and post-Td humoral antibody responses observed in our study subjects may be representative of the tetanus and diphtheria vaccination status of adult males living in rural Indonesia. Tetanus and diphtheria are important causes of death among infants and children in this nation [14], a situation that relates directly to the immunity and carrier status of adult Indonesians. The Indonesian National Immunization Program has targeted children and pregnant women since 1977 [15]. Many adults, particularly males, remain at risk of tetanus and may be reservoirs of diphtheria infection. The low frequencies of protective titers we observed at baseline, the magnitude of titers achieved by Td vaccination, and the rapid post-Td vaccination fall in titer are not indicative of a population that received full tetanus immunization as children. Paradoxically, however, virtually all subjects manifested protective anti-diphtheria titers at baseline and developed impressively high prolonged post-Td titers as a result of the single inoculation. On the basis of the low anti-tetanus responses seen and trends in coverage by the National Immunization Program, we do not believe that the high anti-diphtheria titers in these young adults resulted from prior vaccination. We suspect that toxigenic and nontoxigenic strains of *Corneybacterium diphtheriae* circulate naturally in the community, widely present in adults as inapparent, chronic, and immunizing infections.

In summary, long-term and concurrent malaria prophylaxis with primaquine or chloroquine did not inhibit the development and duration of humoral immune responses following Td vaccination. The initially high anti-tetanus and anti-diphtheria IgG titers observed in primaquine users complement their cellular responses against tetanus and may engender a more effective longer-lasting immunity. These results constitute an additional measure of safety assurance to support regulatory agency evaluation of primaquine for malaria prophylaxis.

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Risk Factors for Repeated Gonococcal Infections: San Francisco, 1990–1992

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Gonococcal (GC) infections are very common and are sustained by a core group of persons who often have repeated GC infections. Identifying individual risk factors for repeated GC infection is essential so that infection control programs can develop better strategies for decreasing the incidence of GC infection. A case-control study among high-risk persons found that being African American, having previous chlamydia infection, and having less than a high-school education were associated with repeated GC infections. Remarkably, measures of sexual behavior and access to health care were not associated with repeated GC infections. These findings suggest that among high-risk persons, the community prevalence of GC infection is more important in predicting risk for repeated GC infections than individual behavior. Interventions should include continued use of resources in high-prevalence communities and better understanding of the roles social and economic discrimination play in the risk for GC infections.

In the absence of an effective vaccine, the prevention of gonococcal (GC) infections relies on identifying and treating infected persons and in modifying behaviors that place individuals at risk for GC infection. Despite existing prevention efforts, GC infections remain a significant problem in the United States and throughout the world [1, 2]. GC infections are among the most common of bacterial diseases, are frequently reacquired, and are sustained by a core group of persons who engage in high-risk behaviors and who have a high prevalence of infection [2–13]. Therefore, an increased understanding of risk factors, particularly behavioral risk factors, within the core group of persons with repeated GC infections is essential so that infection control programs can develop better strategies for decreasing the incidence of GC infection.

During the 1970s, studies defined the importance of repeated GC infections and described the demographic characteristics of persons who have repeated GC infections [3–9]. Factors associated with repeated infections included younger age, black race, being single, having less than a high school education, having a history of a previous sexually transmitted disease (STD), and having other concomitant STDs. The usefulness of some of these studies was limited in assessing currently important behavioral factors, such as partner selection and coupling dynamics, and in determining the role of access to health care because of study design or scope of data collection.

In 1990, during an increase in the number of cases of GC infection in San Francisco, we undertook a case-control study to identify patterns that might be associated with repeated GC infections. We assessed demographic and new behavioral factors, such as frequency, duration, and types of sexual partnerships; health-care utilization and perceived access; and illicit substance use. This report describes the results of that study.

Methods

Patients. From August 1990 through June 1992, we prospectively examined the San Francisco City and County STD control database each day to identify new records of case-patients with repeated GC infections. A case of repeated GC infection was defined as a heterosexual patient age 15–24 years who was identified in the database with a current diagnosis of gonorrhea and either a history of gonorrhea within the past 2 years or a history at any time of pelvic inflammatory disease (PID) associated with gonococcal infection. Control-patients were selected from persons who were identified in the database with a current diagnosis of gonorrhea within 2 weeks of the date of diagnosis of the case-patient and who had no known history of gonorrhea. Recruiters were blinded to the case-control status of the patient. A disease-control investigator made up to three attempts to recruit patients into the study by letter, telephone, and field visits. Case-patient and control-patient

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This study was approved by the University of California, San Francisco, Committee on Human Research. Informed consent was obtained from all study participants.

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histories were confirmed by questioning the patient and examining individual patient medical records. Patients unable to be located or ineligible were replaced with the next available patient in the database. The demographic characteristics of case- and control-patients enrolled in the study were compared with the demographic characteristics of all cases from the STD control database during the same time period.

Data collection. Information about patient demographics, health, sexual behavior, and illicit substance use patterns was recorded during a private face-to-face interview by trained interviewers using a structured questionnaire. Questions about health-care access included frequency of medical visits, setting of provider, and medication usage. Data on sexual and drug use behavior were collected for patient and sex partners for the 2 months before the interview and for the patient's lifetime. Patients were asked to categorize their sex partners as (1) main or regular partners and (2) non-main or casual partners. Information about sex partner demographics, health, sexual behavior, and illicit substance use patterns was collected for each category of sex partner.

Statistical methods. Comparisons of cases and controls were done by t and χ^2 tests for association. Variables that were not normally distributed were also examined by the Mann-Whitney U test. Fisher's exact test was used for contingency tables with cells smaller than 5 cases. $P < .05$ was considered statistically significant for bivariate comparisons.

Multivariate analysis involved the selection of all variables identified in the bivariate analysis as significantly distinguishing between groups at $P < .10$. To facilitate comparison between odds ratios, patients were classified by age as to whether they were older than the median of 20 years. Logistic stepwise regression with backward elimination was used to identify variables uniquely related to group membership at $P < .10$.

Results

During the study period, 185 persons with newly diagnosed GC infection were enrolled: 94 had a history of previous GC infections and 91 had no prior history of GC infection. The mean age (±SD) of the study population was 20 (±2) years; 65% were male, 80% were African American, 7% were Hispanic, 5% were white, 1% were Asian, and 7% were classified as other. Most repeat cases (76%) had 1 previous infection; the maximum was 26. Demographic comparisons of study enrollees with all reported cases of GC infection in San Francisco County during this same period demonstrated that the demographics of the study population were similar. During the enrollment period, 2634 cases of GC infection were reported in San Francisco residents ages 15–24 years. Of these, 57% were male, 64% were African American, 11% were Hispanic, 11% were white, 3% were Asian, and 11% were other. Mean age (±SD) was 20 (±2.5) years.

Patients with repeated GC infections did not differ from patients with first-time GC infection with respect to any of the following variables: number of medical visits in the past year or 5 years, ability to identify a regular doctor, having a partner with an STD, being told by the health department they had been exposed to an STD, smoking, douching, number of years of sexual activity, number of lifetime sex partners, number of sex partners in the previous 2 months, frequency of having a new sex partner in the previous 2 months, frequency of condom use in the previous 2 months, frequency of condom use by any partner type (main or non-main partner) in their most recent sexual encounter, reporting intoxication during sex by any partner type in the previous 2 months, or receiving money for sex in the previous 2 months.

Patients with repeated GC infections were more likely to be African American but less likely to be employed or have a high school education (table 1). Patients with repeated GC infections were also more likely to report a history of chlamydia infection or another STD, including syphilis, chancroid, PID, nonspecific urethritis, mucopurulent cervicitis, trichomoniasis, venereal warts, and herpes, and were more likely to have received drugs for sex. Subanalysis revealed that of the 9 persons who reported receiving drugs for sex, 7 recalled that the drug was crack cocaine.

Cases and controls did not differ by age of their partner when the data were stratified by patient's sex. Cases and controls differed significantly, however, regarding the race of their partner, particularly a non-main partner. Patients who had repeated GC infections were more likely to identify their most recent partner as being of their own race than were patients with first-time GC infection ($P = .04$). Patients who had repeated GC infections were more likely to have African American partners (odds ratio = 2.3; 95% confidence interval, 1.1–4.7). However, when race of partner was stratified by patient's race, this was no longer statistically significant.

Table 1. Selected characteristics of study patients with and without repeated gonococcal (GC) infections, San Francisco, 1990–1992.

Patient characteristic	Gonococcal infection %		
	First-time ($n = 91$)	Repeated ($n = 94$)	P
Demographics			
African American	71	88	.004
Currently employed	37	24	.04
⩾High school education	65	50	.04
Sexually transmitted disease (STD) history			
Ever having sex partner inform patient of STD	22	33	.09
Report of prior chlamydia infection	15	37	.03
Report of prior STD other than GC infection	35	57	<.001
Reported receiving drugs for sex	2	10	.03
Race			
Any partner African American	36	56	.01
Non-main partner who is same as patient	67 ($n = 57$)	82 ($n = 68$)	.04

Table 2. Adjusted odds ratios (ORs) in the multivariate logistic regression model for repeat gonococcal infections, San Francisco, 1990–1992.

Factor	Adjusted OR (95% confidence interval)
African American	2.96 (1.29–6.77)
Completed high school	0.47 (0.23–0.95)
Previous chlamydia infection	2.94 (1.38–6.26)

Multivariate analysis was used to identify factors associated with patients with repeated GC infections. Logistic regression analysis (table 2) indicated that patients with repeated GC infections were more likely to be African American and have had a previous chlamydia infection but were less likely to have completed high school.

Discussion

The results of this study suggest that among high-risk persons, measures of sexual behavior, such as number of partners, number of new partners, years sexually active, and condom use, are not associated with repeated GC infections. In addition, no associations were found between measures of health care access (e.g., number of medical visits within the past year or past 5 years and being able to identify a regular provider) and having repeated GC infections. On the other hand, patients with repeated GC infections were more likely to be African American, to have a history of chlamydia infection, and to have not completed high school.

The lack of findings related to individual sexual behavior and the persistent association of African American race and lack of high school education with repeated GC infections in this study and others [4–6, 9, 11] underline the importance of community prevalence in a person's risk for repeated GC infections. The majority of subjects in this study were African American. African Americans in San Francisco are likely to be lower in economic status than whites, thus the findings that being African American increased the odds of repeated infections and higher education decreased the odds suggest that economic status and discrimination can play a part in the risk for repeated GC infections. Analysis of our results by race allowed for the identification of disadvantaged groups (African Americans who have increased rates of GC infection) and showed that the risk of reinfection is high in African Americans independent of specific sexual behaviors and utilization of health services.

Individuals are likely to choose partners who have characteristics similar to their own because of the increased likelihood that persons with similar sociodemographic characteristics will congregate in social situations where partnering might occur [11, 14, 15]. If a given community has an increased prevalence of infection, it follows that a person who has sex within this community will have an increased risk of infection and, in this study, increased risk of repeated GC infections.

The association of lack of high school education with repeated GC infections may suggest factors relating to sexual mixing, but it also could suggest economic and social limits that affect partner selection. Further study is needed to clarify this association. The association with previous chlamydia infection and repeated GC infections might be explained by sexual mixing within a group at high risk for STDs. In addition, previous chlamydia infection might be a marker for biologic susceptibility for GC infection.

This study was limited by several factors. The classification of case or control status relied on retrospective data obtained from self-report, provider records, and the STD control morbidity database. It is unlikely that cases were misidentified since case status depended on documented GC infection, but control status may have been misidentified by absence of documented GC infection or by a patient's unawareness of previous GC infection. This could have led to differential misclassification in which controls were more similar to cases and could have resulted in underestimates of the observed associations or an inability to find true associations. A second limitation was the relative homogeneity of the study population, which could have prevented the detection of other factors associated with repeated GC infections.

These findings add to those of previous studies showing that certain minority populations and those with less formal education are at increased risk for repeated GC infections [4–6, 9]. Our study indicates that areas for intervention should include continued targeting of prevention and control resources to African Americans in San Francisco and increased efforts to understand the roles that social and economic discrimination play in partner selection and individual risk for STDs.

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