Humoral Immunity to Cytomegalovirus and Chronic Graft-Versus-Host Disease

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Abstract

Human cytomegalovirus (HCMV) is an important cause of morbidity and mortality in patients with chronic graft-versus-host disease (cGVHD), but the underlying mechanisms are not understood. The aim of this investigation was to determine whether humoral immune responses to the HCMV antigens were quantitatively different in hematopoietic cell transplant (HCT) recipients who developed cGVHD from those who did not. Antibodies to HCMV and its proteins UL94 and UL70 were quantitated in 79 cGVHD and 30 non-cGVHD patients by enzyme-linked immunosorbent assays (ELISAs). Mean levels of antibodies to the whole HCMV and to its protein UL94 were not significantly different between the cGVHD and the non-cGVHD subjects. However, the levels of antibodies to HCMV UL70 were significantly higher in non-cGVHD subjects than in those with cGVHD (20.91 ± 15.63 versus 15.00 ± 10.35 ng/mL; $p = 0.03$). This suggests that anti-UL70 antibodies might play a protective role in the development of cGVHD.

Introduction

THE PATHOPHYSIOLOGY OF CHRONIC GRAFT-VERSUS-HOST DISEASE (cGVHD)—a serious and potentially fatal complication of hematopoietic cell transplant (HCT)—is poorly understood. The clinical and immunological manifestations of cGVHD are remarkably similar to those of systemic sclerosis (SSc; scleroderma), an autoimmune disease characterized by inflammation and fibrosis of the skin and visceral organs (1). In fact, murine sclerodermatous GVHD is one of the animal models of human SSc (2). In addition to the known T-cell-mediated abnormalities, the two diseases also share certain autoantibody profiles (3,4). Infection with human cytomegalovirus (HCMV) may be yet another common link between cGVHD and SSc. HCMV infection has been shown to be associated with an increased risk of cGVHD (5). A well-documented property of HCMV is its ability to infect endothelial cells (6–9), and endothelial dysfunction in cGVHD is similar to that seen in SSc. We and others have shown that levels of antibodies to the whole HCMV and to some of its proteins were significantly higher in SSc patients than in control subjects (10–13), and more importantly, antibodies to the HCMV protein UL94 caused apoptosis of endothelial cells (11). Another HCMV protein relevant to both SSc and cGVHD is UL70. It is structurally homologous to topoisomerase I (14), an enzyme that contributes to the genomic stability by regulating

winding and unwinding of DNA. Antibodies to topoisomerase I are present in a significant proportion of patients with the diffuse form of SSc (15), and they have also been detected in cGVHD patients (3).

The aim of this investigation was to determine whether the levels of antibodies to the whole HCMV and its proteins UL94 and UL70 were significantly different in HCT recipients who developed cGVHD from those who did not.

Materials and Methods

The study population consisted of 109 patients who underwent HCT at Stanford University. Of these, 79 developed cGVHD, the majority (60) having the extensive form of the disease. Patient characteristics divided by HCT donor-recipient HCMV seropositivity are shown in Table 1. The study was approved by the Institutional Review Boards of Stanford University and the Medical University of South Carolina.

Inactivated, density-gradient-purified HCMV strain AD 169 was obtained from Virusys Corporation (Taneytown, MD). Round-bottom 96-well plates (Greiner Bio-One, Monroe, NC) were coated with 1 μ g/mL of whole HCMV in carbonate bicarbonate buffer (pH 9.6), and incubated at 37°C for 1 h. The plates were washed with phosphate-buffered saline (pH 7.40, containing 0.05% Tween 20 (PBS-T), and incubated with 1% BSA in PBS-T. The plates were washed

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TABLE 1. CHARACTERISTICS OF THE PATIENTS STUDIED

	D-/R- (n=21)	D-/R+ (n=32)	D+/R- (n=13)	D+/R+ (n=43)
Age	45 (28-64)	51.5 (19-65)	50 (24-63)	49 (20-66)
Gender				
Male	11 (52)	19 (59)	11 (85)	25 (58)
Female	10 (48)	13 (41)	2 (15)	18 (42)
Underlying disease type				
Myeloid cancer	10 (19)	14 (26)	7 (13)	22 (42)
Lymphoid cancer	10 (19)	18 (35)	6 (12)	18 (35)
Others	1 (25)	0 (0)	0 (0)	3 (75)
cGVHD status				
Non-cGVHD	4 (19)	8 (25)	4 (31)	14 (33)
cGVHD	17 (81)	24 (75)	9 (69)	29 (67)
Limited	4 (19)	7 (22)	3 (23)	5 (11)
Extensive	13 (62)	17 (53)	6 (46)	24 (56)

Age is expressed in median years followed by the range in parentheses. For each variable presented, the number of patients in each group is followed by the percentage in parentheses.

D-/D+, donor HCMV negative/positive; R-/R+, recipient HCMV negative/positive; HCMV, human cytomegalovirus; cGVHD, chronic graft-versus-host disease.

and incubated with serially-diluted patient serum and incubated for 30 min at 37°C. The plates were further washed and probed with anti-human IgG HRP conjugate (Sigma-Aldrich, St. Louis, MO), along with HRP substrate, hydrogen peroxide, and tetramethylbenzidine (TMB) as chromogenic substrate. The reaction was stopped by the addition of 2N HCl, and the absorbance values were monitored at 450 nm on a BioTek ELISA reader (BioTek, Winooski, VT).

UL94-specific ELISA was performed as previously described (12). UL70-specific ELISA was performed using a peptide described by Muryoi *et al.* (14). Studies by these workers found that human topoisomerase 1 shares five sequential residues (aa 121-126) with the UL70 protein of HCMV. Only one-third of its amino acids are hydrophobic, so it was coupled to KLH to increase its binding to microtiter plate wells for use as antigen. Briefly, a 15-amino acid consensus UL70 antigenic peptide (CMDQDDGYFMHRRLLP), with an additional N-terminal cysteine residue coupled to keyhole limpet hemocyanin was synthesized by Peptide 2.0 (Chantilly, VA), and used as antigen. The plates were coated with 1 µg/

mL of this conjugate and incubated for 1 h at 37°C and ELISA was continued as above. One positive serum sample was used as reference for each antigen. The amount of IgG present in this sample was measured by ELISA using purified human whole IgG as standard. The levels of antibodies to these antigens were then extrapolated from these references run side by side along with the samples. All antibody determinations were performed blinded to the donor-recipient HCMV seropositivity status.

Comparisons between the cGVHD and non-cGVHD groups were performed using analysis of covariance (ANCOVA) models with the log₁₀-transformed variables (anti-HCMV, anti-UL70, and anti-UL94 antibodies) serving as the dependent variables. Tests of normality confirmed that the normality assumptions of the *t*-test were valid. The HCT source (peripheral blood versus bone marrow) is known to influence the HCMV viral load (16), which could in turn influence the magnitude of antiviral antibody responsiveness. Because of the potential for donor and/or recipient HCMV seropositivity status and the HCT source to influence antibody levels, the ANCOVA models were adjusted for these variables, thus providing an estimate of the independent association between cGVHD status and antibody levels. Sensitivity analyses were conducted to see if the observed associations were dependent on the subjects' disease stages (i.e., extensive or limited). All tests were two-tailed, and statistical significance was defined as *p* < 0.05.

Results

Table 2 presents the analyses of the levels of antibodies to the whole HCMV, and to the HCMV UL94 and HCMV UL70 proteins, between cGVHD and non-cGVHD subjects. After adjusting for covariates (including donor and recipient cytomegalovirus status and specimen source), mean levels of antibodies to the whole HCMV were not significantly different between the cGVHD and the non-cGVHD subjects (9.49 ± 11.14 versus 8.19 ± 7.83 µg/mL; *p* = 0.76). Similarly, adjusted mean levels of antibodies to the HCMV antigen UL94 antigen were also not significantly different between the two groups (313.58 ± 514.50 versus 326.15 ± 521.80 ng/mL; *p* = 0.70). However, the levels of antibodies to HCMV UL70 were significantly higher in non-cGVHD subjects than in those with cGVHD (20.91 ± 15.63 versus 15.00 ± 10.35 ng/mL; *p* = 0.03). When analyses were conducted that compared controls only to cGVHD patients with "limited" disease (*n* = 19), the differences in log₁₀ HCMV UL70 remained significant (mean difference 0.19 units; 95% CI 0.03, 0.35; *p* = 0.03). Similarly, when analyses were conducted that compared controls only to cGVHD patients with "extensive" disease (*n* = 60), the

TABLE 2. COMPARISONS OF THE LEVELS OF ANTIBODIES TO THE WHOLE HCMV AND HCMV UL94 AND HCMV UL70 PROTEINS BETWEEN cGVHD AND NON-cGVHD SUBJECTS

Variable	cGVHD (n=79): mean (SD)	Non-cGVHD (n=30): mean (SD)	p Value ^a
Anti-HCMV (µg/mL)	9.49 (11.14)	8.19 (7.83)	
Log ₁₀ anti-HCMV	0.67 (0.59)	0.64 (0.60)	0.76
Anti-HCMV UL94 (ng/mL)	313.58 (514.50)	326.15 (521.80)	
Log ₁₀ anti-HCMV UL94	2.10 (0.60)	2.16 (0.56)	0.70
Anti-HCMV UL70 (ng/mL)	15.00 (10.35)	20.91 (15.63)	
Log ₁₀ anti-HCMV UL70	1.09 (0.30)	1.22 (0.29)	0.03

^a*p* Values were obtained from analysis of covariance models that adjusted for donor and recipient cytomegalovirus status (i.e., present versus absent), and specimen source (i.e., peripheral blood versus bone marrow).

HCMV, human cytomegalovirus; cGVHD, chronic graft-versus-host disease; SD, standard deviation.

differences in log₁₀ HCMV UL70 remained significant (mean difference 0.13 units; 95% CI 0.00,0.26; $p=0.05$). After similar subgroup analyses, the differences in anti-HCMV and anti-UL94 levels remained non-significant.

Discussion

Higher anti-UL70 antibody levels in non-cGVHD subjects compared to cGVHD subjects suggest that the presence of these antibodies may be protective. The observed lack of association between the other two antibodies measured—anti-HCMV and anti-UL94—and the disease status is not clear. One explanation could be that the HCMV-spurred/induced pathway to cGVHD pathogenesis is viral epitope- and disease-dependent. This is plausible, as HCMV is associated with diverse forms of human diseases—such as scleroderma (11), glioblastoma (17), and retinitis (18)—that are unlikely to involve the same pathway to the disease phenotype. We do not know the mechanism underlying the possible protective role of anti-UL70 antibodies in the pathogenesis of cGVHD, but it is tempting to speculate on a mechanism involving molecular mimicry—immune response against antigens shared by the host and a virus. As mentioned earlier, UL70 is structurally homologous to topoisomerase I, which regulates winding and unwinding of DNA. Cross-reacting anti-UL70 antibodies could bind to topoisomerase I, thereby interfering with DNA replication and the division of virally-infected cells. This would impede cell-to-cell transmission of the virus. The level of protection offered by anti-UL70 antibodies is likely to depend on host genetic factors. The prevalence of anti-topoisomerase I antibodies is known to be genetically controlled (15), and the generation of antibodies to UL70 might similarly be restricted by the host immune response genes.

If the results presented here were confirmed by an independent investigation, they could lead to HCMV UL70-based adoptive immunotherapy for HCMV disease in HCT recipients. Numerous researchers have investigated the role of cellular immunity to HCMV in HCT recipients, but there is a paucity of studies on the role of humoral immunity (19). Additional studies are needed.

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Author Disclosure Statement

No competing financial interests exist.

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