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Polymorphisms in the Genes Encoding Chemokine Receptor 5, Interleukin-10, and Monocyte Chemoattractant Protein 1 Contribute to Cytomegalovirus Reactivation and Disease after Allogeneic Stem Cell Transplantation

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We analyzed 90 polymorphisms in 17 genes related to immune function for association with human cytomegalovirus (HCMV) reactivation and disease in patients after allogeneic stem cell transplantation. We found relevant markers (i) in CCR5 and IL-10 genes conferring a higher risk for the development of HCMV disease and (ii) in the MCP1 gene associated with HCMV reactivation. Testing of high-risk patients for the presence of these single-nucleotide polymorphisms might be useful for individualizing antiviral prophylaxis.

Single-nucleotide polymorphisms (SNPs) may influence the rate and regulatory dynamics of gene transcription, the stability of the mRNA, and the production and biological activity of the resulting protein (14). Previously, it has been demonstrated that SNPs are associated with susceptibility to inflammation and autoimmune diseases. In particular, SNPs in genes coding for cytokines or cytokine receptors were reported to be associated with increased risks for infections (2).

Human cytomegalovirus (HCMV), a ubiquitous betaherpesvirus, interacts with various target cells during acute and latent infections. HCMV infection is one of the most challenging complications after allogeneic stem cell transplantation (alloSCT). About half of the patients with HCMV infection not receiving antiviral chemotherapy will develop HCMV disease (7).

In this study, we genotyped polymorphisms (n = 90) of the loci of the immune-related genes encoding interleukin-4 (IL-4) (5q31.1), IL-6 (7p21), IL-10 (Iq31-q32), IL-12B (5q31-q33), IL-18 (11q22.2-22.3), gamma interferon (IFN-γ) (12q14), tumor necrosis factor alpha (TNF-α) (6p21.3), small inducible cytokine A20 (CCL20) (2q33-q37), monocyte chemoattractant protein 1 (MCP1) (17q11.2-q12), chemokine CXC motif ligand 10 (CXCL10) (4q21), chemokine receptor 1 (CCR1) (3p21), CCR5 (3p21), CCR6 (6p27), CCR7 (17q12-q21.2), intercellular adhension molecule 1 (ICAM1) (19p13.3-p12.3), Toll-like receptor 2 (TLR2) (4p32), and TLR4 (9q32-q33) for an association between defined SNPs and asymptomatic HCMV reactivation (DNAemia) and HCMV disease. Three groups of patients were analyzed: patients with HCMV reactivation, (group I), patients with HCMV disease (group II), and patients without HCMV reactivation (group III). HCMV reactivation and disease was defined by Ljungman et al. (11). HCMV disease includes pneumonia, gastrointestinal disease, hepatitis, retinitis, nephritis, cystitis, myocarditis, and pancreatitis. In a series of case-control-type analyses, group III served as controls and will be referred to as the HCMV-negative group, whereas joined groups I and II will be called HCMV positive. The following comparisons were performed: group I versus controls, group II versus controls, group I versus group II, and groups I and II combined versus controls. All 154 patients received an alloSCT.

Between 1994 and 2003, EDTA-anticoagulated whole-blood samples (5 to 10 ml) were collected around 30 days after alloSCT at the University of Perugia, Perugia, Italy (n = 8); the Fred Hutchinson Cancer Research Center, Seattle, Wash. (n = 57); Huddinge University Hospital, Stockholm, Sweden (n = 7); the University of Mannheim, Mannheim, Germany (n = 13); and Medical Hospital II, Tübingen, Germany (n = 69).

For more extensive typing of SNPs in IL-10 and MCP1 genes and for analysis of clinical risk factors, additional patients from the University of Essen, Essen, Germany (n = 74), and Medical Hospital II, Tübingen, Germany (n = 14), were evaluated. DNA was extracted by standard methods based on spin column technology and frozen at −20°C or −80°C until further analysis. Retrospective analysis was approved by the local ethics committees.

Genotyping of 83 patients (with HCMV reactivation) and 71 controls (no HCMV reactivation) was performed. The combination of a seronegative donor and a seronegative patient was excluded from the analysis. All donors and patients, including

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TABLE 1. Comparison of characteristics of patient controls and patients with CMV reactivation and/or disease

Characteristic	Patient controls (not reactivated)	Patients with CMV reactivation and/or disease
Median age, yr (range)	39 (17–71)	43 (19–55)
No. of males/no. of females	49/34	38/33
No. with chronic myeloproliferative disorders[a]	7	9
No. with AML, ALL, SAA, NHL, or MDS[a]	66	51
No. with $CD34^+$ cell selection	47	40
No. with acute GvHD, grades II to IV	66	72
No. undergoing corticosteroid therapy (>2 mg/kg of body wt)	81	64

[a] For 21 patients, no underlying disease could be determined retrospectively. AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; SAA, severe aplastic anemia; NHL, non-Hodgkin's lymphoma; MDS, myelodysplastic syndrome.

the individuals from the United States, were Caucasians. Details of the patient cohorts are shown in Table 1.

Genotyping was performed by pyroseqencing on the PSQ HS96A system (Biotage AB, Uppsala, Sweden) or on the ABI 7900 ABI PRISM sequence detection system with TaqMan Assay-By-Design and Assay-On-Demand (Applied Biosystems). Procedures were performed according to the manufacturers' instructions.

For single-marker association analysis, we used the Armitage trend test (15). The level of significance was set to 0.05. In order to detect effects of population substructure or admixture, as well as systematic genotyping artifacts, all control and patient groups were tested for all markers on Hardy-Weinberg equilibrium. With low genotype counts, an exact test (5) was used; a chi-square test with one degree of freedom was used otherwise.

For the three genes (the CCR5, MCP1, and IL-10 genes) with at least one highly significant association ($P < 0.001$) for a single marker, additional haplotype analyses were performed with the programs EHplus (19) and FAMHAP (1). The Monte Carlo permutation method with a minimum of 100,000 replicates was carried out to evaluate empirical P values of the log-likelihood ratio tests comparing the HCMV-positive group versus the HCMV-negative control group for each possible haplotype.

Additionally, we determined whether the following clinical risk factors might be associated with DNAemia and disease: selection of $CD34^+$ cells prior to alloSCT ($n = 87$); corticosteroid therapy with a dosage of >2 mg/kg of body weight ($n = 145$); severe acute graft-versus-host disease (GvHD), grades II to IV ($n = 138$); and chronic GvHD ($n = 83$).

By the Armitage trend test, we found no significant association for 71/80 markers and the occurrence of HCMV reactivation or disease (for details, see http://klinik.uni-wuerzburg.de/img/ejbfile/jcm164705supplement.pdf?id=3982). Ten markers in the IL-4 and IL-6 genes had to be excluded from the statistical analysis because of a low genotyping call rate. However, 9/80 markers showed a significant association with HCMV reactivation and/or disease ($P < 0.05$). These markers are located in the CCR5, MCP1, and IL-10 genes. Five of these nine markers showed P values of <0.01, indicating a highly significant association with either CMV reactivation or disease (Tables 2 and 3).

The P values shown in Tables 2 and 3 are single point and unadjusted. As a consequence of multiple testing, a significance level of 0.05 might be regarded too liberal. However, there has been an ongoing discussion about the necessity of P value adjustment in exploratory epidemiological studies since the appearance of Rothman's publication (13) that has been continued recently (6). These issues are not expected to be

TABLE 2. Association between genetic polymorphisms in the CCR5, MCP1, and IL-10 genes and HCMV reactivation and disease[a]

Gene and dbSNP[c] no.	Nucleotide position (allele)[b]	Control vs HCMV positive	Control vs reactivation	Control vs disease	Reactivation vs disease
CCR5 (3p21)					
rs2734648	−2554 (**G**/T)	0.182	0.671	0.014	0.017
rs1799988	−2135 (C/T)	0.623	0.896	0.534	0.687
rs1800023	−2086 (**A**/G)	0.208	0.497	0.011	**0.009**
rs746492	2919 (A/C)	0.346	0.965	0.150	0.205
rs17141079	10176 (A/**T**)	0.728	0.214	0.119	0.018
MCP1 (17q11.2-q21.1)					
rs1024611	−2581 (**C**/T)	0.034	0.029	0.272	0.570
rs4586	901 (C/T)	0.585	0.388	0.747	0.353
rs13900	1543 (C/**T**)	**0.006**	0.021	0.014	0.476
IL-10 (1q31-q32)					
rs1800893	−1387 (**A**/G)	0.854	0.248	0.153	**0.009**
rs1800896	−1117 (A/**G**)	**0.007**	0.147	**0.001**	0.016
rs1800871	−854 (C/T)	0.571	0.499	0.907	0.745
rs1518111	1136 (A/G)	0.496	0.134	0.870	0.194
rs1554286	1548 (C/T)	0.383	0.099	0.794	0.092
rs3024492	1668 (**A**/T)	0.484	0.420	0.097	0.035
rs1878672	2068 (C/**G**)	**0.010**	0.115	**0.003**	0.060

[a] Patients in the control group did not show HCMV reactivation. Significant P values (<0.05) obtained by the Armitage trend test are underlined, and P values of <0.01 are in boldface. Positions of the SNPs in the respective genes were determined at http://snpper.chip.org/.

[b] In significant markers, risk alleles are in boldface.

[c] dbSNP, SNP database.

TABLE 3. Association between defined clinical risk factors and HCMV reactivation and disease[a]

Clinical parameter	Control vs HCMV positive	Control vs reactivation	Control vs disease	Reactivation vs disease
Acute GvHD	0.031	1.000	**0.001**	**<0.001**
Chronic GvHD	1.000	0.059	0.231	**0.003**
Corticosteroids (>2 mg/kg)	0.143	0.036	0.452	0.959
CD34$^+$ cell selection	0.242	0.037	**0.001**	**<0.001**
CMV serostatus,[b] +/+	**0.005**	**<0.001**	0.250	0.390
CMV serostatus, +/−	**0.006**	**0.005**	0.046	0.552
CMV serostatus, −/+	**0.007**	**<0.001**	0.124	0.817

[a] Patients in the control group did not show HCMV reactivation. Significant *P* values (<0.05) obtained by the Armitage trend test are underlined, and *P* values of <0.01 are in boldface. Positions of the SNPs in the respective genes were determined at http://snpper.chip.org/.
[b] Serostatus of the donor/serostatus of the recipient.

resolved soon, and with all relevant data reported, final judgment is left to the reader.

Except for marker rs13900 in the MCP1 gene and rs3024496 and rs1518111 in the IL-10, gene, when tested in patients with CMV disease, all markers in the CCR5, IL-10, and MCP1 genes were found not to deviate from Hardy-Weinberg equilibrium, and no deviation from Hardy-Weinberg equilibrium was found in the control group. Thus, there was no indication for population substructure or admixture and for systematic genotyping artifacts.

When individuals with HCMV DNAemia were compared with those with no reactivation regardless of disease, four markers showed a significant association (in the MCP1 and IL-10 genes). Interestingly, both markers of the MCP1 gene were significantly associated with HCMV reactivation; by contrast, neither of the markers in the CCR5 and IL-10 genes were associated with HCMV reactivation. Thus, we presume that HCMV reactivation is associated with polymorphisms in MCP1 gene, whereas disease might be related to polymorphisms in the CCR5 and IL-10 genes.

The haplotype analyses confirmed the association between HCMV and the IL-10 and MCP1 genes showing a corrected *P* value of 0.0026 for the IL-10 gene and of 0.0447 for the MCP1 gene. In the IL-10 gene, the lowest *P* value (0.0001) of any marker combination was achieved for the markers rs3024496, rs1800871, and rs1800893. Among several conspicuous haplotypes, the most frequent risk haplotype was CGA, with an odds ratio of 1.65. In the MCP1 gene, the lowest *P* value (0.0182) corresponded to the two-marker combination of rs1024611 and rs4586 with an odds ratio of 1.70 for the risk haplotype GC. There was no significant haplotypic association when markers within the CCR5 gene were analyzed.

Numerous reports exist that polymorphisms in these genes influence the outcome and course of infectious diseases. The promoter of the IL-10 gene contains several polymorphisms from which the SNPs at positions −1082, −819, and −592 have been intensively analyzed (16). It has been shown that these polymorphisms determine whether individuals develop severe clinical symptoms after herpesvirus infections (8). We found significant associations between SNPs at positions −1117 (rs1800896), 1668 (rs3024492), and 2068 (rs1878672) and the development of HCMV disease.

As the MCP1 gene plays a critical role by directing the migration of monocytes to sites of infection and the promoter has been shown to contain binding sites for the transcription factor NF-κB, modifications in this gene are likely to influence innate immunity (17). One of our significant markers of the MCP1 gene (−2581, C/T) is localized in this region. Furthermore, we demonstrated that the marker rs2734648 (−2554, G/T) in the promoter of the CCR5 gene influences susceptibility to HCMV. In parallel, O'Brien and Moore showed that this SNP accelerates the progression of AIDS (12).

HCMV has developed various strategies for immune evasion to establish a lifelong persisting infection in the host. It is remarkable that HCMV targets exactly those genes which showed susceptibility to the virus in our analysis. For example, HCMV codes for an IL-10 homolog which might allow the virus to suppress cellular immune responses (10). In addition, HCMV encodes four viral chemokine receptors allowing depletion of chemokines from the medium, such as the receptor that binds MCP1 with high affinity (3). Furthermore, the down-regulation of the cell surface expression of CCR5 reduces the migration of various immune effector cells to sites of inflammation (18). This highlights the link between HCMV-mediated manipulation of IL-10, MCP1, and CCR5 activity and its ability to escape the immune system.

Analyzing the clinical parameters (Tables 2 and 3), we found an association between CD34$^+$ selection and HCMV reactivation ($P = 0.037$) or HCMV disease ($P = 0.001$). The enrichment of CD34$^+$ cells prior to transplantation leads to a delayed immune reconstitution because of a parallel depletion of B and T cells in the transplant. Furthermore, severe acute GvHD was significantly related to HCMV disease ($P = 0.001$) while chronic GvHD was significantly associated with HCMV disease only if reactivation versus disease was compared ($P = 0.034$). Treatment with corticosteroids led to an increase in HCMV reactivation ($P = 0.036$). These clinical risk factors correspond to previously published observations (4, 9).

Further studies based on the markers described and including a much larger cohort of patients are being coordinated.

In conclusion, screening of alloSCT patients for the presence of defined polymorphisms may help to predict the individual risk of HCMV reactivation and disease, thus justifying intensified antiviral prophylaxis.

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REFERENCES

1. **Becker, T., and M. Knapp.** 2004. A powerful strategy to account for multiple testing in the context of haplotype analysis. Am. J. Hum. Genet. **75:**561–570.
2. **Bidwell, J., L. Keen, G. Gallagher, R. Kimberley, T. Huizinga, M. F. McDermott, J. Oksenberg, J. McNicholl, F. Pociot, C. Hardt, and S. D'Alfonso.** 2001. Cytokine gene polymorphism in human disease: on-line databases, supplement 1. Genes Immun. **2:**61–70.
3. **Casarosa, P., M. Waldhoer, P. J. LiWang, H. F. Vischer, T. Kledal, H. Timmerman, T. W. Schwartz, M. J. Smit, and R. Leurs.** 2005. CC and CX3C chemokines differentially interact with the N terminus of the human cytomegalovirus-encoded US28 receptor. J. Biol. Chem. **280:**3275–3285.
4. **Einsele, H., H. Hebart, C. Kauffmann-Schneider, C. Sinzger, G. Jahn, P. Bader, T. Klingebiel, K. Dietz, J. Löffler, C. Bokemeyer, C. A. Müller, and L. Kanz.** 2000. Risk factors for treatment failures in patients receiving PCR-based preemptive therapy for CMV infection. Bone Marrow Transplant. **25:**757–763.

5. **Elston, R. C., and R. Forthofer.** 1977. Testing for Hardy-Weinberg equilibrium in small samples. Biometrics **33:**536–542.
6. **Feise, R. J.** 2002. Do multiple outcome measures require P-value adjustment? BMC Med. Res. Methodol. **2:**8–11.
7. **Hebart, H., and H. Einsele.** 2004. Clinical aspects of CMV infection after stem cell transplantation. Hum. Immunol. **65:**432–436.
8. **Hurme, M., M. Haanpaa, T. Nurmikko, X. Y. Wang, M. Virta, T. Pessi, S. Kilpinen, J. Hulkkonen, and M. Helminen.** 2003. IL10 gene polymorphism and herpesvirus infections. J. Med. Virol. **70:**48–50.
9. **Kim, D. H., J. G. Kim, N. Y. Lee, W. J. Sung, S. K. Sohn, J. S. Suh, K. S. Lee, and K. B. Lee.** 2004. Risk factors for late cytomegalovirus infection after allogeneic stem cell transplantation using HLA-matched sibling donor: donor lymphocyte infusion and previous history of early CMV infection. Bone Marrow Transplant. **34:**21–27.
10. **Kotenko, S. V., S. Saccani, L. S. Izotova, O. V. Mirochnitchenko, and S. Pestka.** 2000. Human cytomegalovirus harbors its own unique IL10 homolog (cmvIL10). Proc. Natl. Acad. Sci. USA **15:**1695–1700.
11. **Ljungman, P., P. Griffiths, and C. Paya.** 2002. Definitions of cytomegalovirus infection and disease in transplant recipients. Clin. Infect. Dis. **34:**1094–1097.
12. **O'Brien, S. J., and J. P. Moore.** 2000. The effect of genetic variation in chemokines and their receptors on HIV transmission and progression to AIDS. Immunol. Rev. **177:**99–111.
13. **Rothman, K. J.** 1990. No adjustments are needed for multiple comparisons. Epidemiology **1:**43–46.
14. **Sachidanandam, R., D. Weissman, S. C. Schmidt, J. M. Kakol, L. D. Stein, G. Marth, S. Sherry, J. C. Mullikin, B. J. Mortimore, D. L. Willey, S. E. Hunt, C. G. Cole, P. C. Coggill, C. M. Rice, Z. Ning, J. Rogers, D. R. Bentley, P. Y. Kwok, E. R. Mardis, R. T. Yeh, B. Schultz, L. Cook, R. Davenport, M. Dante, L. Fulton, L. Hillier, R. H. Waterston, J. D. McPherson, B. Gilman, S. Schaffner, W. J. Van Etten, D. Reich, J. Higgins, M. J. Daly, B. Blumenstiel, J. Baldwin, N. Stange-Thomann, M. C. Zody, L. Linton, E. S. Lander, D. Altshuler, and the International SNP Map Working Group.** 2001. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature **409:**928–933.
15. **Sasieni, P. D.** 1997. From genotypes to genes: doubling the sample size. Biometrics **53:**1253–1261.
16. **Turner, D. M., D. M. Williams, D. Sankaran, M. Lazarus, P. J. Sinnott, and I. V. Hutchinson.** 1997. An investigation of polymorphism in the interleukin-10 gene promoter. Eur. J. Immunogenet. **24:**1–8.
17. **Ueda, A., Y. Ishigatsubo, T. Okubo, and T. Yoshimura.** 1997. Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-κB sites and NF-κB/Rel subunit specificity. J. Biol. Chem. **272:**31092–31099.
18. **Varani, S., G. Frascaroli, M. Homman-Loudiyi, S. Feld, M. P. Landini, and C. Soderberg-Naucler.** 2005. Human cytomegalovirus inhibits the migration of immature dendritic cells by down-regulating cell-surface CCR1 and CCR5. J. Leukoc. Biol. **77:**219–228.
19. **Zhao, J. H., D. Curtis, and P. C. Sham.** 2000. Model-free analysis and permutation tests for allelic associations. Hum. Hered. **50:**133–139.