Pattern of the Epitope-Specific IgG/IgM Response against Human Cytomegalovirus in Patients with Multiple Myeloma

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Human cytomegalovirus (HCMV) is a member of the herpesvirus family and represents a major human pathogen causing severe disease in newborns and immunocompromised patients, e.g., organ transplant recipients and patients with AIDS. HCMV is widespread throughout the population worldwide. The seroprevalence in adults varies from 50 to 100% (1). Although the infection is rarely of significance in individuals with a competent immune system, immune control cannot achieve clearance of the virus. Thus, reactivation during immunosuppression leads to recurrent episodes of disease. HCMV disease is responsible for a number of syndromes, including acute mononucleosis, retinitis, colitis, esophagitis, pneumonia, hepatitis, and meningoencephalitis (2). Furthermore, congenital infections remain a major concern, despite the relatively low incidence (estimated range, 0.15% to 2.4%), because of the severity of the long-term sequelae, e.g., hearing loss and mental deficits (3). Although HCMV is not currently known to be an agent for causing human cancer, recent reports provide evidence that HCMV is associated with human malignancies. Antigen expression, as well as nucleic acids, has been detected in a large proportion of malignant tumors: colon cancers, prostate cancers, glioblastomas, medullablastomas, and breast cancers (4–7). It has been suggested that HCMV leads to the dysregulation of multiple pathways involved in oncogenesis (8, 9). Further investigations are needed to determine the exact role of HCMV in these tumors.

In this study, the humoral immune response to HCMV in patients with multiple myeloma (MM) was determined. MM is a B-cell neoplasia in which malignant plasma cells accumulate in the bone marrow and secrete large amounts of a monoclonal antibody. MM accounts for approximately 1% of neoplastic diseases and 13% of hematologic cancers (10, 11). In Western countries, the annual age-adjusted incidence is 5.6 cases per 100,000 persons (10, 11). The median age at the time of diagnosis of MM is approximately 70 years; 37% of MM patients are younger than 65 years, 26% are between the ages of 65 and 74 years, and 37% are 75 years of age or older (10, 11). Despite significant progress in the treatment of MM due to the improved efficacy of autologous and allogeneic stem cell transplantation and introduction of the proteasome inhibitor bortezomib and the immunomodulatory drugs thalidomide, lenalidomide, and dexamethasone, a large percentage of MM patients, unfortunately, experience relapse (12–14).

Myeloma patients display cellular and humoral immunodeficiencies, which increase following conventional as well as high-dose chemotherapy, and these constitute important predisposing factors for opportunistic infections (15).

Human cytomegalovirus is an important pathogen after allogeneic transplantation, which is rarely performed in MM patients. Few studies have examined HCMV reactivation after autologous peripheral blood stem cell transplantation for the treatment of MM. In a study by Kim et al. (16), the majority of patients were HCMV seropositive, and HCMV viremia was frequently detected in this group. No primary HCMV infections were identified. HCMV reactivation was more common in recipients of tandem transplantation than in recipients of a single transplantation (16). In addition, patients who developed HCMV viremia were more likely to have received conditioning therapy with melphalan, bortezomib, dexamethasone, and thalidomide than those without HCMV reactivation.

In this study, we analyzed the HCMV-specific humoral immune response of patients with MM in comparison to that of healthy donors. An accredited immunoblot test (recomBlot CMV;
Mikrogen Diagnostik, Neuried, Germany) was used for detection of the antibody response against HCMV. This assay includes recombinant immunodominant epitopes for three structural proteins, glycoprotein B and the tegument proteins pp150 and pp65, and three nonstructural proteins, immediate early protein 1 (IE1), the processivity factor pUL44, and the single-stranded binding protein pUL57 (CM2 is a fusion of the pUL44 and pUL57 epitopes). The determination of the kinetics and pattern of antibody response may lead to a better understanding of the pathogenesis of HCMV infection in MM patients. Here we present the findings of an initial study validating this assay for its ability to address this important point in the future.

MATERIALS AND METHODS

Patients. In this study, a total of 20 patients with MM and, as a control group, 20 healthy donors (without oncogenic or other preexisting conditions) were included. Nineteen MM patients had in the past received therapy with bortezomib in combination with dexamethasone. The disease of one patient was characterized as stage IA; thus, treatment was not recommended. Nine patients received high-dose chemotherapy (melphan-prednisolone) with autologous peripheral blood stem cell transplantation (Table 1).

Immunoblotting. For determination of different antibody patterns, the recomblot CMV IgG or IgM test from Mikrogen Diagnostik was used. This qualitative test allows assignment of an antibody response to specific antigens in human serum and plasma by using immunoblot strips. These immunoblot strips contain recombinant polypeptides from IE1 (53 kDa), tegument protein pp150 (50 kDa), processivity factor pUL44 and single-stranded binding protein pUL57 (45 kDa; CM2), tegument protein pp65 (31 kDa), and two epitopes of glycoprotein B (gB1, 25 kDa; gB2, 18 kDa). After 3 min of incubation, only low-avidity antibodies were removed by the three washing steps, whereas high-avidity antibodies were stably bound. Both strips were incubated with peroxidase-conjugated anti-human IgG for 45 min at RT. After addition of the substrate solution (5 to 10 min), specific bound antibodies were detected.

Evaluation of band intensity and interpretation of IgG/IgM detection. In all tests, a weak positive control was included and the bands were evaluated according to the results for this control. Interpretation of IgG detection was as follows: negative, no HCMV-specific bands; -/+; bands with very weak intensity; positive, antibodies against pp150 with weak intensity or greater (+), a negative result for antibodies against pp150 but well visible antibodies against CM2 (2+), and a 2+ result plus 1 further band of weak intensity (+); and borderline, all other constellations.

In general, antibodies against pp150, pp65, IE1, and CM2 were observed in the early phase of infection, while antibodies against gB1 and gB2 were normally detectable after 6.8 weeks of infection. High titers of IgG against all HCMV proteins in combination with positive IgM titers could be an indication of reactivation.

Interpretation of IgM detection was as follows: for high avidity, the band intensity on the avidity strip was not reduced for at least two antibodies (IE1, pp150, CM2) and indicated a former infection (past); for low avidity, the band intensity of at least two antibodies (IE1, pp150, CM2) on the avidity strip was reduced by ≥50% of the level on the IgG strip and indicated a primary infection (fresh); for intermediate avidity, the band intensity of at least one antibody (IE1, pp150, CM2) on the avidity strip was reduced by ≥50% of the level on the IgG strip and indicated a recent infection.

ELISA. Routine serological analysis of HCMV IgM was performed by using an enzyme-linked immunosorbent assay (ELISA), the CMV-IgM-ELA test PKS medac (medac, Hamburg, Germany) on the basis of the instructions given by the manufacturer. Briefly, 50 μl patient serum (1:100 diluted) and the negative and positive controls were loaded onto a 96-well microtiter plate and incubated for 1 h at 37°C. After three washing steps, 50 μl of the enzyme-labeled antigen (CMV-IgM-ELA) was added and the plate was incubated for 1 h at 37°C. Following the washing steps, 50 μl tetramethylbenzidine substrate was added and the plate was incubated for 30 min at 37°C. The reaction was stopped by addition of 100 μl stop solution, and a photometric reading at 450 nm was performed to measure the wavelength (reference wavelength, 620 to 650 nm). The mean optical density (OD) value of the negative control had to be <0.1, and that of the positive control had to be >0.8. Samples with OD values of <0.9 were reported to be negative, those with OD values of ≥1.1 were reported as positive.
to be borderline, those with OD values of >3.5 were reported to be weak positive, and those with OD values of >3.5 were reported to be positive.

**Ethics statement.** Patient sera were collected in accordance with the Declaration of Helsinki and with the approval and under the guidelines of the Charité Ethics Commission (EA1/277/12).

**Biostatistical analysis.** In order to determine the comparability of the results for our patient cohort, statistical analyses were performed. The results obtained from a paired Student’s t test were used to calculate significance. A P value of ≤0.05 was considered significant.

### RESULTS

**Features of patients with HCMV infection.** Analyses of our collected patient data showed that 8 out of 9 patients who underwent high-dose chemotherapy with autologous stem cell transplantation either had an HCMV infection in the past or had a recent or fresh infection (Table 1; see Table 3). Since HCMV seroprevalence is age dependent, statistical analyses were performed with data for our patient cohort. These analyses showed that there was no significant difference in the age groups of the healthy donors and the MM patients (Table 2).

**Pattern of IgG antibodies against HCMV.** To determine the pattern of the immune response against HCMV in multiple myeloma patients, a sensitive recombinant immunoblot test (recomBlot CMV; Mikrogen) was used. Out of 20 patient serum samples, 16 (80%) showed an anti-HCMV IgG response (Table 2). In the control group consisting of sera from healthy donors, an IgG immune response of 65% was detected (Table 2). The pattern of IgG antibodies varied in each patient. All of the seropositive patients and healthy donors had antibodies against the tegument protein pp150 (p150), 68% had antibodies against the immediate early protein (IE1), and 68% had antibodies against CM2 (epitopes from the processivity factor pUL44 and the single-stranded DNA

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**Table 2 Characteristics of HCMV-seropositive patients**

| Group and HCMV Ig status | No. (%) of individuals | Median (range) age (yr) |
|--------------------------|------------------------|------------------------|
|                          | Total      | Male    | Female  |                      |
| MM patients              |            |         |         |                      |
| HCMV IgG positive        | 16 (80)    | 9 (45)  | 7 (35)  | 62.8 (53–77)         |
| HCMV IgM positive        | 11 (55)    | 6 (30)  | 5 (25)  | 67.0 (56–76)         |
| Healthy donors           |            |         |         |                      |
| HCMV IgG positive        | 13 (65)    | 7 (30)  | 6 (25)  | 62.2 (46–82)         |
| HCMV IgM positive        | 7 (35)     | 3 (15)  | 4 (20)  | 60.4 (46–72)         |

*For MM patients and healthy donors, the P values for the IgG/IgM ratio were 0.488 and 0.599, respectively. The P value for MM patients/healthy donors was 0.259.*

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**FIG 1** IgM (A) and IgG (B) patterns of antibodies in sera from MM patients and healthy donors. Samples from MM patients and healthy donors were analyzed for their responses to six different HCMV epitopes. Analyses were repeated once.
Immune Response against HCMV in MM Patients

binding protein pUL57). The response against the tegument protein pp65 (p65) was higher in the MM patients than in the healthy donors (75% and ~60%, respectively). The antibody responses against glycoproteins were approximately 70% against gB2 and 100% against gB1 (Fig. 1B).

In addition, to determine the time that the infection occurred, the avidity of IgG antibodies was analyzed. Subsequent comparison of two test strips provided the avidities of the antibodies and an estimate of the infection stage. In general, an acute reaction was characterized by low-avidity antibodies to IE1, pp150, and CM2, whereas high-avidity antibodies characterized former infections. Examples of results for fresh, recent, and past infections are shown in Fig. 2. While the majority of our patients (n = 10) had a former infection, a fresh HCMV infection was detected in four patients and a recent infection occurred in two patients (Table 3; Fig. 2). In the group of healthy donors, only one fresh infection was determined (Table 4).

**Determination of IgM-positive sera.** In order to identify IgM-positive patient sera, immunoblot analysis with the recomBlot CMV IgM test was used. We detected an IgM immune response in 55% (11 out of 20) of our MM patients (Table 5) and 35% (7 out of 20; Table 6) of the healthy donors. The quality of the immune response to IgM of MM patients and healthy donors was similar. Interestingly, the most prominent response in both groups was against HCMV tegument protein pp150 (Fig. 1A, p150), whereas only 27% (MM patients) and 28% (healthy donors) recognized the epitopes of CM2 (Fig. 1). A very weak response against pp65 was observed in only two MM patients and one healthy donor (Fig. 1A, p65). To verify these results, routine serological analysis using an IgM ELISA (CMV-IgM-ELA test PKS; medac) was performed. According to the recommendations of the manufacturer, none of the sera tested showed an IgM immune response against HCMV.

**DISCUSSION**

Several recent reports indicated a role of HCMV in patients with malignancies. In order to determine the effect in patients with

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![Image](http://cvi.asm.org)
multiple myeloma, analyses of the humoral immune response against HCMV were performed.

By using a recombinant immunoblot test, we detected an IgG immune response against HCMV in 80% of multiple myeloma patients. Interestingly, 65% of healthy donors were IgG seropositive. In general, the seroprevalence in adults in Europe is about 40 to 60%. It is known that in some risk groups, e.g., HIV-infected patients, the seroprevalence is over 90%. One could hypothesize that the weaker general condition of MM patients might lead to a higher risk for HCMV infection. In addition, the major antibody response in MM patients was against the epitope of the tegument protein pp150.

Interestingly, in 55% of the MM patients and 35% of the healthy donors, an IgM response was detected in the recombinant immunoblot test. This seroprevalence is relatively high compared to that found by Dollard et al. (17). This could be due to the fact that the difference in age between the cohort of Dollard et al. (17) and our cohort was 2 decades. It might be that the seroprevalence of IgM increases rapidly after a certain decade of age. However, the results of the IgM ELISA were negative. One explanation would be that a whole-virus preparation instead of recombinant epitopes is used in the ELISA. The amount of proteins in virus preparations expressing immunodominant epitopes is less than the amount of recombinant proteins expressing immunodominant epitopes. In addition, the immune response against HCMV nonstructural proteins (IE1, pUL44, pUL57), known to detect early responses, could not be analyzed (18). In regard to the tested MM patients, the HCMV-specific IgM response was directed against the pp150 and the pUL44/57 epitopes. It has been shown that although higher humoral immune responses to epitopes on natural proteins were detected in more patients, it seems that the responses to immunodominant epitopes on recombinant proteins detected are more related to the HCMV load during infection (19). In addition, Müller et al. (20) demonstrated an IgM response against HCMV pp150 in one-third of clinically asymptomatic renal transplant patients. Additional epitopes were recognized in patients with clinical manifestations. Therefore, the authors suggested that the severity of HCMV infections correlates with the humoral immune response against increasing numbers of epitopes (20).

**TABLE 4 IgG pattern and avidity data for antibodies from healthy donors**

| Patient no. | Age (yr) | IE1 | p150 | CM2 | p65 | gB1 | gB2 | IgG test result | IE1 | p150 | CM2 | p65 | gB1 | gB2 | Avidity | Comment |
|-------------|---------|-----|------|-----|-----|-----|-----|----------------|-----|------|-----|-----|-----|-----|---------|---------|
| 12-029      | 49      |     | +    |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         | Fresh   |
| 12-032      | 59      |     | +/−  |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         | Past     |
| 12-033      | 63      |     | +    |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         | Past     |
| 12-040      | 73      |     | +    |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         | Past     |
| 12-043      | 72      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-045      | 71      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-048      | 71      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-038      | 49      |     | +    |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |

**TABLE 5 IgM pattern of antibodies from patients with MM**

| Patient no. | Age (yr) | IE1 | p150 | CM2 | p65 | gB1 | gB2 | IgM test result |
|-------------|---------|-----|------|-----|-----|-----|-----|----------------|---------|
| 12-001      | 73      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-003      | 67      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-004      | 74      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-006a     | 59      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-008b     | 49      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-010      | 67      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-013b     | 77      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-019      | 53      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-020      | 73      |     |      |     |     |     |     | Negative       | IE1 |     |     |     |     |     |         |         |
| 12-005a     | 56      |     | +    |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         |         |
| 12-016      | 71      |     |      |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         |         |
| 12-002      | 76      |     | +    |     |     |     |     | Positive       | IE1 |     |     |     |     |     | +/−     |         |
| 12-007      | 67      |     | +    |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         |         |
| 12-009      | 73      |     |      |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         |         |
| 12-011      | 59      |     |      |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         |         |
| 12-012      | 69      |     | +    |     |     |     |     | Positive       | IE1 |     |     |     |     |     | +/−     |         |
| 12-014      | 73      |     |      |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         |         |
| 12-015      | 72      |     |      |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         |         |
| 12-017      | 64      |     |      |     |     |     |     | Positive       | IE1 |     |     |     |     |     |         |         |
| 12-018      | 61      |     | +    |     |     |     |     | Positive       | IE1 |     |     |     |     |     | 2+      |         |

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3+/−, very weak intensity (intensity weaker than that of the pp150 band of the control); +/−, weak intensity (intensity the same as that of the pp150 band of the control); 2+, well visible (intensity greater than that for the pp150 band of the control); 3+, very strong intensity; blank cell, negative result.
It is known that IgM antibodies were generated after primary infection with human cytomegalovirus; however, those can also appear during reactivation or reinfection (21). Therefore, HCMV IgM antibodies could not be used as the only serological indicator of a primary infection. Recently, Dollard et al. (17) demonstrated that measurement of IgG avidity combined with IgM antibody levels provides a reliable diagnostic marker of primary HCMV infection. A low HCMV IgG avidity together with high HCMV IgM antibody titers was associated with primary infection in patients. In the case of our MM patients, similar observations were found in four cases. However, sera from two of the MM patients diagnosed with fresh cases of infection and one of the healthy donors had a low IgG avidity but no IgM antibodies. Interestingly, all of the MM patients with fresh cases had received an autologous peripheral blood stem cell transplantation in the past. Since the rate of infection among these patients was not increased compared to that among healthy people, this observation is not due to impaired immune cells.

In order to provide useful clinical information, it is recommended that both analyses be used in conjunction. This reliable diagnostic tool will give the opportunity to understand the association of infection with tumor-related factors (e.g., anti-MM therapy, progression) and to identify those MM patients who will most likely benefit from early antiviral therapy in the future. In our knowledge, this study is the first one to show the humoral immune response to HCMV in MM patients. Future analyses will be undertaken to determine the effect of HCMV on tumor progression and pathogenesis.

**ACKNOWLEDGMENTS**

We thank Ina Woskobojnik for performing the immunobLOTS. We are grateful to all donors for their agreement to participate in this study. This work was funded in part by the Forschungsförderung der Charité to G.P. and E.B.

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