Discordant cellular and humoral immune responses to cytomegalovirus infection in healthy blood donors: existence of a Th1-type dominant response

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Abstract

Previous studies have documented discordant cellular and humoral immune responses to subjects exposed to HIV-1, and that the nature of such responses may determine susceptibility and resistance to disease. We determined whether there is a spectrum of cellular versus humoral immunodominant responses to cytomegalovirus (CMV) infection. Blood samples from 50 healthy blood donors were tested for anti-CMV IgG antibodies and for proliferative responses of peripheral blood mononuclear cells (PBMC) to CMV antigens. Four patterns of immune responses to CMV were found: no detectable response (30%, Ab–/Tc–), anti-CMV IgG only (28%, Ab+ /Tc–), both anti-CMV IgG and T lymphocyte proliferation to CMV antigens (18%, Ab+ /Tc+), and, interestingly, T lymphocyte proliferation to CMV only (24%, Ab–/Tc+). To determine whether these immunodominant phenotypes correlate with the ability of PBMC to secrete IL-2 and IFN-γ in response to CMV antigens, we found that a greater percentage of individuals with a T cell proliferative response to CMV antigens (Ab+/Tc+ and Ab+/Tc+) responded with increased IL-2 (P = 0.001) and IFN-γ levels (P = 0.002), compared to those without a proliferative response (Ab+/Tc– and Ab+/Tc–). Our data therefore demonstrate that different individuals exhibit different immunodominant patterns of response to CMV. In particular, some individuals who are exposed to CMV fail to develop an antibody response but do develop cellular immunity. Whether these different patterns predict susceptibility or resistance to CMV-induced disease remains to be determined.

Introduction

The type of immune response (cellular or humoral) to an infection can influence whether the host will succeed in eliminating the pathogen, or will develop persistent infection with the establishment of chronic or recurrent disease. Although the humoral arm of the immune system is important mainly for prevention of infection or spread of infection via extracellular compartments, if pathogens gain entry to intracellular sites the cell-mediated immune response becomes essential to pathogen elimination or control. Data compatible with this concept come from studies of infectious diseases such as HIV-1 (1–5), chronic hepatitis B (6) and leishmaniasis (7–9).

Human cytomegalovirus (CMV), like other Herpesviruses, persists in the infected host for life. Under certain circumstances, it can be reactivated to cause clinically important disease. Most known CMV-related diseases occur in immune-compromised patients, such as patients following organ transplantation (10) and AIDS patients (11). Clinically relevant CMV-induced disease, however, may not be limited to immune-compromised subjects, as an increasing body of data suggests that CMV may contribute to the development of vascular diseases such as re-stenosis following coronary angioplasty (12,13) and atherosclerosis (14–17).

Previous studies have demonstrated that the cell-mediated
immune response to CMV is of critical importance in eliminating the virus from the host in a murine model (18). Although studies in immunocompetent humans are not available, it was found that patients with AIDS who had a cellular-mediated immune response to CMV were relatively resistant to CMV-induced retinitis, while patients lacking this response were susceptible (11). We hypothesized that certain immunocompetent individuals infected with CMV lack an efficient cell-mediated immune-surveillance system targeted to CMV and thereby have an impaired capacity to eliminate the virus or to prevent its reactivation from latency.

As a first step in testing the validity of this hypothesis, we determined whether a spectrum of humoral versus cellular responses to CMV infection exists in immunocompetent individuals, and whether discordant responses of these arms of the immune system can occur in the same individual, as has been demonstrated in the response to HIV-1S (1–5) and more recently to hepatitis C virus (19). The demonstration that such disparities in the immune response to CMV do in fact exist would be of importance as it would raise new paradigms for understanding individual variations in the susceptibility versus resistance to chronic disease related to CMV and perhaps even more generically to chronic disease that may be induced by a broad array of pathogens.

Methods

Blood donors

Fifty healthy individuals were entered into this study. They volunteered under a National Heart, Lung and Blood Institute IRB-approved protocol to donate blood to the Transfusion Medicine Department, National Institute of Health. These healthy blood donors consisted of 76% men; 64% were White, 34% Black and 2% Asian. Their ages ranged from 28 to 62 years (mean 40 and median 39). They were randomly chosen independent of age, sex or race. None of the blood donors had antibodies against HIV or hepatitis C. None had acute CMV infection with systemic viremia.

Isolation of peripheral blood mononuclear cells (PBMC)

PBMC from each blood sample were separated on lymphocyte separation medium (Organon Teknika, Durham, NC) by centrifugation at 1800 r.p.m. for 25 min at room temperature. The separated cells were collected and washed twice in PBS (Gibco, Grand Island, NY). The number of viable cells was determined by Trypan blue exclusion using a hemocytometer. PBMC were then cryopreserved in aliquots in liquid nitrogen until used.

Antibody status to CMV

Serum collected for the detection of antibodies was frozen at −80°C. CMV IgG antibodies were determined using an ELISA kit (CYTOMEGELISA II; Biowhittaker, Walkersville, MD). Antibody results were calculated from standard curves provided by the manufacturer. The threshold value for a ‘positive’ result was determined prospectively: an ELISA value <0.25 U was considered a negative result and a value of ≥0.25 U was considered positive, indicating prior exposure to CMV. Samples for anti-CMV IgG antibodies were tested in triplicate and in two separate experiments.

CMV antigen preparations

The Towne strain of human CMV was obtained from ATCC (Rockville, MD) and grown in human fibroblasts, HEL299 (ATCC), for preparation of the viral antigens. Growth media consisted of minimum essential medium (Gibco) supplemented with 2% fetal bovine serum and antibiotics. Virus titer was measured on HEL299 cells. The protocols for CMV antigen preparations have been published (11,20,21). Briefly, CMV antigens were prepared with (i) heat-inactivated CMV (1 h at 56°C) that was obtained from supernatants of CMV-infected fibroblasts (final concentration of virus was 105 p.f.u. before inactivation), (ii) cell lysates of CMV-infected fibroblasts by repeated freezing and thawing, and (iii) 0.08% glutaraldehyde-fixed, CMV-infected fibroblast cells. Both cell lysates and fixed cells were prepared from 2–106/ml cells by infecting a 90% confluent monolayer of human fibroblasts with CMV at a m.o.i. of 10. Cells were collected by centrifugation when they showed 50% cytopathic effect. The large stocks were aliquoted and stored at −70°C. Controls for the CMV antigens were obtained from non-infected fibroblasts (mock-infected cells), prepared exactly as described for CMV-infected cells.

T lymphocyte proliferation

T lymphocyte proliferative responses were performed in 96-well flat-bottom plates (Costar, Cambridge, MA). An aliquot of 100 μl of PBMC (3×105/ml) was added to each well. PBMC were cultured at 37°C with 5% CO2 in RPMI 1640 (Gibco) containing 5% human AB serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and HEPES buffer, with or without antigen stimulation. After 5 days of culture, or 3 days for phytohemagglutinin (PHA) stimulation, each well was pulsed with 1 μCi of [3H]thymidine, and harvested 18 h later. Thymidine incorporation was determined using a model LS1801 β-spectrometer (Beckman Instruments, Fullerton, CA). All samples were assayed in triplicate and expressed as mean c.p.m. The data are presented as stimulation index (c.p.m. of cultures in the presence of CMV antigens divided by c.p.m. of cultures in the absence of CMV antigens). If a sample responded to two out of three CMV antigen preparations (heat-inactivated supernatants of CMV-infected fibroblasts, CMV-infected cell lysates or fixed CMV-infected fibroblasts) by a stimulation index >4, the response was considered positive. Positive controls included: (i) 3 days of stimulation with PHA (1:200; Gibco), (ii) influenza A/Bangkok RX73 (flu; grown in embryonated eggs and used as infectious allantoic fluid at an infectivity of 2×104 tissue culture infectious dose50/ml at a final dilution of 1:1000), (iii) Candida antigen (Greer Laboratories, Lenoir, NC; final dilution of 20 mg/ml), and (iv) a pool of irradiated (5000 rad) PBMC from three unrelated healthy donors (2×106/ml). Negative control included: (i) supernatants, cell lysates and fixed cells from mock-infected fibroblasts prepared exactly as described for CMV-infected cells, and (ii) RPMI media control.

Detection of cytokine production after in vitro antigenic stimulation

Using the same protocol for CMV antigen-induced T lymphocyte proliferation, supernatants of the cultures were collected.
Results

To determine whether different individuals have different immunodominant humoral and cellular responses to CMV antigens, blood samples from healthy blood donors were tested for anti-CMV IgG antibodies and for a T lymphocyte proliferative response to CMV antigens by [3H]thymidine uptake. Figure 1 shows the patterns found. Of the 50 individuals, nine (18%) had both anti-CMV IgG antibodies and a T cell proliferative response to CMV antigens (referred to as the Ab+/Tc− subgroup). Fourteen (28%) who had anti-CMV antibodies did not show a CMV-induced T lymphocyte response (referred to as the Ab+/Tc−/Tc+ group). There were 15 individuals (30%) who were negative for both antibodies and T lymphocyte proliferation to CMV (referred to as the Ab−/Tc−/Tc− group). Interestingly, 12 (24%) individuals who did of the CMV major immediate early gene (MIE, UL123) and from the CMV late pp150 gene (UL32) were used for PCR.

Table 1. Cell-type specific proliferative response of PBMC to CMV antigens

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<td>Ab+/Tc−</td>
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CMV−: no exposure to CMV antigens. CMV+: exposure to CMV antigens.

Ab+/Tc−: individuals with antibody-positive but T cell proliferation-negative responses to CMV antigens. Ab−/Tc+: individuals with antibody-negative but T cell proliferation-positive responses to CMV antigens. Data are expressed as the mean ± SEM.

Statistical analysis

Data were analyzed by the t-test, χ²-test or Fisher’s exact test. One degree of freedom χ²-tests were used to test the independence (lack of trend) of the percentage of individuals with increased levels of cytokines from the presence or absence of a positive cellular response to CMV antigen stimulation. All tests were two-tailed. P values less than 0.05 were considered significant.

Results

Patterns of humoral and cellular immune responses to CMV antigens

DNA preparation and PCR analyses

DNA preparation and PCR analyses were performed at BioServe (Laurel, MD) by using the methods as described previously (22,23). DNA was isolated from PBMC samples as well as from mock-infected and CMV-infected human fibroblasts (HEL-299). Analysis was performed in 30 PBMC samples (12 samples from the Ab+/Tc+ group, 14 from the Ab−/Tc− group and two from the Ab−/Tc−/Tc+ group). CMV-specific primers from exons 1 and 2 and 3 were used to amplify DNA prepared from mock- and CMV-infected HEL299 cells was included in each PCR assay as a negative and a positive control respectively. Glucose 6-phosphate dehydrogenase (G6PD) primer from exons 6 and 7 of the G6PD gene was used as a control for the presence of DNA or cDNA in each sample. The PCR amplification products were transferred to a nylon membrane by vacuum blotting of the agarose gel and the DNA was cross-linked to a nitrocellulose filter. Hybridizations with CMV-specific 32P 3′-end-labeled oligonucleotide probes for MIE and pp150 were performed as described previously (23).

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Data were analyzed by the t-test, χ²-test or Fisher’s exact test. One degree of freedom χ²-tests were used to test the independence (lack of trend) of the percentage of individuals with increased levels of cytokines from the presence or absence of a positive cellular response to CMV antigen stimulation. All tests were two-tailed. P values less than 0.05 were considered significant.

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not produce anti-CMV IgG antibodies had positive proliferative responses to CMV antigens (referred to as the Ab+/Tc+ subgroup). In contrast, the T lymphocyte proliferative responses to a mitogen (PHA) and recall antigens (influenza A and Candida antigens) were positive in 48 (96%) and 47 (94%) individuals respectively; the response to these antigens was used as a positive control. No proliferative response was observed in these individuals when their PBMC were stimulated with antigens derived from mock-infected fibroblasts or cultured with medium alone (data not shown).

Identification of CD4+ T cells as the major proliferating cell type in PBMC after CMV antigen stimulation by FACS analysis
To determine whether the CMV-stimulated proliferating cell type mainly belongs to the CD4+ or CD8+ T cell subset, we performed FACS analysis for CD4+ and CD8+ phenotypes of PBMC with and without CMV stimulation in five individuals with Ab+/Tc+ and five with Ab+/Tc− responses to CMV antigens. Table 1 shows the data derived from these studies. Although the background of CD4+ T cell thymidine incorporation was higher in the Ab+/Tc+ group than the Ab+/Tc− group (47.3 versus 28.5%; \( P = 0.02 \)), the only significant increase of CD4+ T cells after CMV antigen stimulation occurred in the Ab+/Tc+ group (\( P = 0.02 \)). As expected, CMV antigen stimulation did not increase CD8+ T cells expansion under the experimental conditions we used.

Cellular immune response against CMV characterized by production of IL-2/IFN-γ
To determine whether individuals with a T cell proliferative response to CMV antigens produce an IL-2 and/or IFN-γ response, we assayed the supernatant of PBMC for IL-2 and IFN-γ production after CMV antigen stimulation. Cytokine levels usually increased within the first 3 days following exposure to CMV antigens and peaked at day 6. PHA stimulation increased IL-2 and IFN-γ levels regardless of CMV seropositivity (data not shown). Table 2 shows that IL-2 and IFN-γ levels increased significantly in response to CMV antigen stimulation in individuals within each of the groups that exhibited either a humoral or cellular immune response to CMV (Ab+/Tc+, Ab−/Tc+ and Ab+/Tc+) at day 6, but not in individuals without immune responses to CMV antigens (Ab+/Tc−). However, a significantly greater percentage of

| Table 2. Mean levels of cytokine concentration in supernatant from PBMC in individuals with different immune responses to CMV antigens |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                | IL-2 (pg/ml)                    | IFN-γ (pg/ml)                   |
| CMV−                           | CMV+                            | \( P \)                          |
| Ab+/Tc−                        | 69.9 ± 24.0                     | 97.7 ± 20.7                     | 0.17                            |
| Ab+/Tc−                        | 77.4 ± 11.9                     | 176.1 ± 24.8                    | 0.002                           |
| Ab+/Tc+                        | 93.0 ± 24.1                     | 230.2 ± 39.3                    | 0.009                           |
| Ab+/Tc+                        | 56.6 ± 13.9                     | 160.3 ± 22.1                    | 0.001                           |
|                                |                                 |                                 |                                 |
| CMV+                           |                                 |                                 |                                 |
| Ab+/Tc−                        | 84.5 ± 30.4                     | 288.5 ± 107.8                   | 0.08                            |
| Ab+/Tc−                        | 138.4 ± 58.2                    | 472.5 ± 104.5                   | 0.001                           |
| Ab+/Tc+                        | 175.4 ± 59.3                    | 687.7 ± 180.0                   | 0.02                            |
| Ab+/Tc+                        | 76.2 ± 18.0                     | 234.1 ± 56.2                    | 0.01                            |

CMV−: no exposure to CMV antigens. CMV+: exposure to CMV antigens.

\( \text{Ab+/Tc−: 15 individuals with antibody-negative and T cell proliferation-negative responses to CMV antigens. Ab+/Tc+: 14 individuals with antibody-positive but T cell proliferation-negative responses to CMV antigens. Ab+/Tc+: nine individuals with antibody-positive and T cell proliferation-positive responses to CMV antigens. Ab+/Tc+: 12 individuals with antibody-negative but T cell proliferation-positive responses to CMV antigens. Data are expressed as the mean ± SEM.} \)
disease (26). These findings raised the possibility that individual variation in the immune response to infection may exist and that such differences may determine susceptibility or resistance to the vasculopathic effects of CMV.

The potential validity of this hypothesis has been reinforced by studies relating in cohorts who were exposed to HIV but remained seronegative. It was demonstrated that a subset of female sex workers (1), homosexual men (27) and HIV-exposed children (3,4,28) who, despite a high exposure rate to HIV-seropositive individuals, had no evidence of HIV infection and did not seroconvert. Lack of infection was associated with a cell-mediated immune response to HIV antigens in the absence of a specific humoral response (as reflected by seronegativity). Similar results were observed in healthy family members of individuals symptomatic with hepatitis C (19).

The results of the present investigation provide evidence that similar divergences in the humoral versus cellular immune responses to CMV infection may also play a role in resistance and susceptibility to CMV-related disease in immunocompetent individuals. We found four immune response subgroups to CMV antigens in healthy blood donors (Fig. 1): (i) neither anti-CMV IgG antibodies nor a T cell proliferative response to CMV antigens (Ab+/Tc+), (ii) antibody responses without T cell responses (Ab+/Tc−), (iii) responses by both arms of the immune system (Ab+/Tc−) and, importantly, (iv) T cell responses in the absence of detectable antibodies to CMV (Ab−/Tc−).

The finding that certain CMV-seronegative individuals have a T cell proliferative response to CMV antigens (Ab+/Tc− subgroup) may be of clinical importance. This is suggested by the finding that these individuals are capable of expanding CD4+ T cells and of secreting IL-2 and IFN-γ in response to CMV antigens. Our findings are consistent with the findings of Clerici et al. (27) that certain persons who are exposed to HIV-1 infection and who do not become infected remain antibody-negative but develop a cell-mediated immune response manifested by secretion of IL-2 by PBMC exposed to certain HIV-1 peptides. In addition, CD4+ cells are essential for eliminating CMV from certain tissues of virus-infected mice. It was also shown that IFN-γ suppresses CMV activity in infected animals (18). Moreover, antigen-induced production of IFN-γ suggests a Th1,1-type of immune response, which in many studies has been identified as the immune component critical for controlling intracellular pathogens (29).

It must be emphasized, however, that the roles of Th1 and Th2 types of immune responses in the control of infectious diseases in humans are complex, and have not yet been fully determined. For example, Th1- and Th2-dominant responses have been suggested not only to provide different modalities of protection against infection, but also to play a critical role in the development and/or maintenance of other pathological conditions (30). Thus, although the existence of immunodominant cellular versus humoral responses to CMV infection in immunocompetent individuals likely has important clinical implications, the precise effects of these disparate responses remain to be elucidated.

It should be stressed that we could detect neither IL-4 nor IL-10 in any of the samples with or without CMV antigen stimulation when we assayed the PBMC supernatant for IL-4 and IL-10 production. This contrasts to the results found when assaying for IFN-γ and IL-2. The reasons for these findings could be: (i) CMV (Towne strain) antigen may fail to stimulate production of certain cytokines from PBMC of our study subjects, (ii) the ELISA kits used for IL-4 and IL-10 may be of limited sensitivity or (iii) high-affinity IL-4 and IL-10 receptors may be expressed on peripheral blood mononuclear cells after CMV antigen stimulation to which the cytokines would bind and therefore lead to falsely low values.

Previous studies suggested that detection of CMV DNA sequences in circulating monocytes by PCR might be a sensitive assay to determine CMV activity (22,23). If this were the case, it would have been interesting to correlate such a finding with immune response phenotype in our cohort. However, we did not find CMV DNA in any of PBMC samples we analyzed. The reasons for the difference between our investigation and previous studies are uncertain.

In summary, our results indicate that there is a spectrum of humoral versus cellular immune responses to CMV infection in immunocompetent individuals. These findings, when considered in conjunction with those from the studies discussed above, raise the possibility that there may be an association between the pattern of immune response and either resistance or susceptibility to the pathogenic influences of CMV. If this proves to be true, the possibility of novel therapeutic strategies arise, as it might be feasible to favorably alter disease outcome by directing attempts to change the immunodominant phenotype from one that increases disease susceptibility to one that promotes resistance (31,32).

Abbreviations

- CMV: cytomegalovirus
- G6PD: glucose 6-phosphate dehydrogenase
- PBMC: peripheral blood mononuclear cell
- PHA: phytohemagglutinin
- Tc: T cell

References

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