

*Review*

## Immunosuppressive Agents and Cytomegalovirus Infection

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**Abstract.** Cytomegalovirus (CMV) infection is the major infectious complication observed after organ transplantation. As rejection episodes always occur in allograft-transplanted recipients, various kinds of immunosuppressive agents are used to control such rejection episodes. Among the commonly used immunosuppressive agents, anti-pan-T cell polyclonal and monoclonal antibody are known to increase the risk of viral infections. New immunological techniques have recently been developed to measure CMV-specific CD4 and CD8 cells by flow cytometry. Using the techniques, high frequencies of specific CD4 and CD8 T cells have been shown to be required to survey the CMV (re)activation in the persistent/latent phase of CMV infection. An excessive T cell depletion by OKT3 would deplete such surveying T cells, thus resulting in the occurrence of CMV-associated diseases.

**Key words:** cytomegalovirus; transplantation; immunosuppression; MHC/antigen-tetramer; virus-specific T cell.

### Introduction

#### *Immunosuppression in organ transplantation*

A transplanted organ from an allogenic donor demonstrates rejection, namely immune-mediated tissue injury. Immunosuppressive agents are thus commonly used in the treatment of such allograft rejection. Azathioprine was first used on a renal transplant recipient in 1961. This was the first case that chemical manipulation was applied to clinical organ transplantation. A combination of azathioprine and corticosteroid was soon thereafter shown to be more effective than azathioprine alone<sup>27</sup>. As a result, this combination has since been widely used in clinical organ transplantation. In the last three decades, various immunosuppressive agents have become available (Table 1). In particular, cyclosporine was discovered in 1972 during screening

studies for anti-microbial properties. This material has drastically increased the number of organ transplants, and has allowed organ transplantation to become a routine clinical procedure. Cyclosporine is now commonly

**Table1.** Commonly used Immunosuppressive agents

| Agents                | Mechanism of action                                |
|-----------------------|----------------------------------------------------|
| Corticosteroid        | blocks cytokine gene transcription                 |
| Azathioprine          | inhibits purine synthesis                          |
| Mycophenolate mofetil | inhibits <i>denovo</i> pathway of purine synthesis |
| ATG/ALG               | depletion of T/whole lymphocytes                   |
| OKT3                  | depletion of pan-T cells                           |
| Chimeric anti-IL-2R   | depletion of activated T cells                     |
| FK506                 | inhibits calcineurin                               |
| Cyclosporine          | inhibits calcineurin                               |

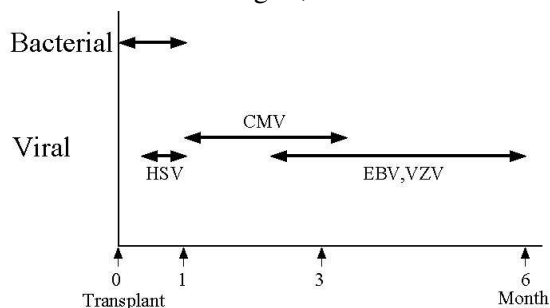
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used in the prevention and treatment of allograft rejection. Antibodies against various subsets of T lymphocytes have also been widely used. Although anti-lymphocyte polyclonal antibodies were used in the early studies, monoclonal antibodies (mAbs) are now used. Among the mAbs, OKT3, a mAb against pan-T cells, is commonly used for the prevention and therapy of rejection. Organ transplantation is now an established clinical procedure. However, as the current immunosuppressive agents lack specificity in immune responsiveness to the allograft, their use is inevitably accompanied by reduced immunity to infections and malignant diseases.

### Immunosuppression and infections

As transplant recipients are in a net state of immunosuppression, their risk of developing infections is extremely high. Although remarkable clinical success has been achieved, infection still remains a major obstacle in organ transplantation. Indeed, infection remains the leading cause of death in transplant recipients. Infections in transplant recipients have several features, as follows:

- infections in the transplant recipients do not randomly occur<sup>17</sup>. They principally occur according to the timetable shown in Fig. 1;



**Fig. 1.** Timetable for the occurrence of infections in transplant recipients. Note that cytomegalovirus (CMV) infections usually occur from 1 to 4 months post-transplant. HSV – herpes simplex virus, EBV – Epstein-Barr virus, VZV – varicella-zoster virus

- the entry sources of infectious microorganisms are air, food, water and secretions in healthy people. In addition, the endogenous organism and the transplanted grafts are potential sources of infectious microorganisms for the transplanted recipients;
- transplant recipients require immunosuppression throughout their lifetimes. As a result, inflammatory responses against infection are attenuated by the immunosuppressive therapy. This sometimes leads to a misdiagnosis of infectious diseases.

### Cytomegalovirus Infection in Organ Transplant Recipients

#### Virology of CMV

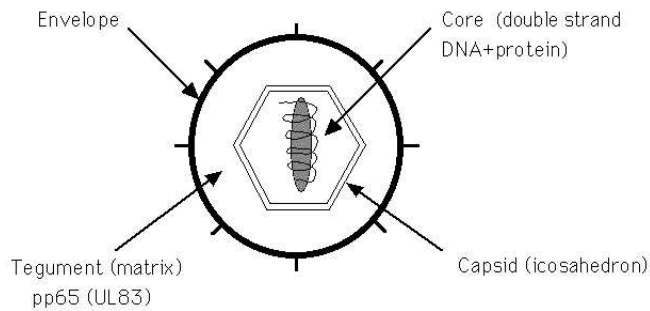
Cytomegalovirus (CMV), a member of  $\beta$ -*Herpesvirinae* of *Herpesviridae*, is a DNA virus which contains 230 kb double-strand DNA. *Herpesviridae* can be divided into three subfamilies (Table 2). The major property of these viruses is their latency, and all of them are important pathogens in organ transplant recipients. Among these microorganisms, CMV is the most important in transplant recipients due to its severity and mortality. Indeed, up to 65% of renal transplant patients<sup>1</sup> and 19–69% of the bone marrow transferred recipients<sup>28</sup> have demonstrated CMV infections. Morphologically, CMV has a typical herpes virion structure, consisting of the materials as shown in below (Fig. 2):

- a core containing a linear, double stranded viral DNA and protein;
- an icosahedral capsid;
- tegument (or matrix);
- an envelope.

The expression of the CMV genome is controlled by a cascade synthesis of viral protein. The first group of the protein to be synthesized is designated as “immediate early” (IE) protein, and the genome responsible for the protein is called the IE gene. The IE protein,

**Table 2.** Human herpesviruses

| Subfamily                       | Official name       | Vernacular name        | Biological properties                          |
|---------------------------------|---------------------|------------------------|------------------------------------------------|
| $\alpha$ - <i>Herpesvirinae</i> | human herpesvirus 1 | herpes simplex virus 1 | latency in neurons                             |
|                                 | human herpesvirus 2 | herpes simplex virus 2 |                                                |
|                                 | human herpesvirus 3 | varicella-zoster virus |                                                |
| $\beta$ - <i>Herpesvirinae</i>  | human herpesvirus 5 | cytomegalovirus        | latency in endothelial and hematopoietic cells |
|                                 | human herpesvirus 6 | HHV-6                  |                                                |
|                                 | human herpesvirus 7 | HHV-7                  |                                                |
| $\gamma$ - <i>Herpesvirinae</i> | human herpesvirus 4 | Epstein-Barr virus     | lymphoproliferative disorder                   |
|                                 | human herpesvirus 8 | HHV-8                  |                                                |



**Fig. 2.** The basic structure of cytomegalovirus (CMV). CMV possesses a typical herpes virion structure; core, capsid, tegument (matrix) and envelope. A major epitope for CD8 T cells in human CMV is pp65, a structural component of tegument

which is not a structural protein, allows the transcription of the mRNA for the early (E) protein. E protein, in turn, allows the transcription of the mRNA for late (L) protein. Both the E and L proteins play a part in the production of daughter virions. It is commonly noted in human individuals that a CMV genome is still present without viral replication, after acute infection. This viral state is called “latent infection”. Under several conditions, the latent CMV virus can restart to produce daughter virions. This state is called “reactivation”.

#### *Epidemiological pattern of CMV infection*

As shown in Table 3, three types of infection occur in CMV infection. A primary infection occurs in a seronegative recipient ( $R^-$ ) who receives blood cells or an organ graft from a seropositive donor ( $D^+$ ). Although any of the three types of CMV infection can provoke symptomatic CMV disease, a primary infection is usually associated with severe symptoms.  $D^+/R^-$  status is, therefore, a high risk factor for the onset of CMV disease. Reactivation occurs in seropositive recipients ( $R^+$ ). In this infection, a latently infected viral DNA genome can be induced to transcript, to produce viral protein and to replicate. Although the mechanism regarding how the latent virus can be reactivated remains an enigma, immunosuppression is the one of the factors known to participate in the reactivation process.

**Table 3.** Type of CMV infection

| Type of infection | Serostatus |           | Risk     |
|-------------------|------------|-----------|----------|
|                   | donor      | recipient |          |
| Primary           | +          | –         | severe   |
| Reactivation      | +/-        | +         | moderate |
| Superinfection    | +          | +         | moderate |

A superinfection can occur in a  $R^+$  who is infected with another strain of CMV.

#### *Pathophysiology of CMV disease*

The clinical manifestation of CMV can be divided into three steps based on the CMV status as follows:

- viral entry/viral reactivation: this step is asymptomatic;
- spread of the virus on a vehicle: the virus spreads using blood cells or endothelial cells as a vehicle. Viremia, which includes antigenemia and DNAmia, can be detected in this step. Ganciclovir is considered to be an effective drug in this step. Such mild manifestations as leukopenia and malaise are frequently noted;
- tissue damage: untreated viremia results in the tissue-invasion of CMV in various organs, including the retina, lung, liver and gastrointestinal tract. Such tissue damage is frequently severe and fatal.

#### **Major Immunosuppressive Agents and CMV Infection**

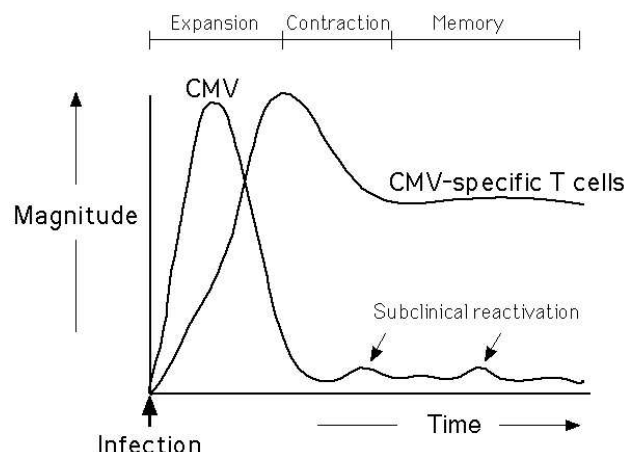
##### *The T cell response in CMV infection*

CMV is a common virus, which is present in the majority of the population. After the primary infection, the virus is rarely eliminated from the infected host and usually establishes a latent or persistent infection. The use of several immunosuppressive agents, which inhibit the host's immune function, apparently increases the risk of CMV infection. The immune response against CMV has so far been mainly examined in human materials and murine models. Although antibodies may help to limit the viral dissemination, cellular immunity remains a major weapon against CMV<sup>8, 28</sup>. MHC-unrestricted natural killer cells and macrophages are the first lines of protection from a primary CMV infection<sup>2, 14</sup>. Recently, such new flow cytometric techniques as the staining of intracellular interferon  $\gamma$  (IFN- $\gamma$ ) and MHC class I/antigen peptide tetramers have been developed<sup>11</sup>. These new techniques for measuring the virus-specific T cells have demonstrated that CD4 and CD8 T cells plays a central role in CMV surveillance.

SESTER et al.<sup>20</sup> demonstrated a high frequency (10–40%) of CMV-specific IFN- $\gamma$ -producing CD4 T cells in seropositive individuals. They reported the specific CD4 T cells to be oligoclonal mature/effector T cells, and that the high frequency of CMV-specific CD4 T was continuously maintained for several years.

As for CD8<sup>+</sup> cytotoxic T lymphocyte (CTL), several antigenic peptides have been reported in human CMV and murine CMV (reviewed in<sup>16</sup>). In murine CMV, IE gene products, which are non-structural components of murine CMV, work as immunogenetic determinants. In human CMV, the 65 kDa phosphoprotein (pp65, UL83), a structural component of tegument (Fig. 2), dominantly serves as a major target epitope for CMV-specific CD8 T cells. Using the HLA class I/pp65-nonapeptide tetramer, GILLESPIE et al.<sup>5</sup> demonstrated that high levels of CMV-specific cells (0.03–5.02% of CD8<sup>+</sup> cells) were noted in immunocompetent donors, and that a substantial proportion of the cells were functionally effective. Using the same HLA/peptide tetramer, extremely high levels of oligoclonal CMV-specific CTLs (0.4–23.0% of CD8<sup>+</sup> cells) have recently been reported in seropositive elderly individuals<sup>10</sup>. The phenotypes of CTL were reportedly those of effector/memory cells. The CTLs enumerated in these reports recognized pp65-nonapeptide presented in a specific class I molecule. However, it is plausible that pp65 is not the only epitope for CMV-specific CTL. Much more specific CD8 T cells than we expected might thus be present in seropositive individuals. These results thus demonstrated that a large population of CMV-specific CD4 and CD8 T cells are present in seropositive individuals. It is not clear how and why such a high frequency of CMV-specific T cells are maintained in seropositive individuals. It has been thought that there are three ways in which CMV keeps T cells activated, these being: 1) activation by CMV, 2) activation by cross reactive antigen, and 3) a bystander activation by cytokines. However, since specific T cells have never been identified in a seronegative individual<sup>9, 11</sup> and CMV antigen does not reportedly contain any broadly activating factors, it is likely that the high frequency of specific T cells is maintained over time by CMV itself. It is therefore suitable to think that persistent CMV with periodic subclinical reactivation boosts the T cell responses (Fig. 3).

We have previously observed similar events in a murine CMV model<sup>22–24</sup>. In this model, a massive release of IFN- $\gamma$  could be elicited in the lungs by *in vivo* T cell stimulation with anti-CD3 mAb, after the clearance of an acute murine CMV infection. Our results suggested that the high frequency of IFN- $\gamma$ -producing cells as maintained in the lungs even after CMV was eliminated. Thereafter, PODLECH et al.<sup>15</sup> reported that IFN- $\gamma$ -producing CD8 T cells persisted in the lungs over time even after the clearance of a murine CMV infection. Those CD8 T cells with memory type phenotypes were capable of exerting effector functions. The



**Fig. 3.** The three phases of the T cell response in cytomegalovirus (CMV) infection. In acute infection, antigen-specific T cells clonally expand (expansion phase). Such cytokines as IL-2 are presumably required for clonal expansion. However, it is usually asymptomatic in this phase for almost all immunocompetent individuals. In the next phase, the contraction phase, the number of CMV drops rapidly, and the clonal expansion of specific T cells is abolished. In the memory phase, CMV is either persistently or latently infected. In this phase, CMV-specific memory/effector T cells are present. IL-2 may not be required for maintaining the specific memory T cells. The periodic subclinical reactivation of CMV may boost T cell response

authors assumed them to be “stand-by” memory-effector cells which work as “guardian” cells to survey the reactivation of CMV.

It is thus plausible that CMV is more frequently reactivated from the persistent/latent state than previously thought. Therefore, a high frequency of specific T cells is required as “guardian” cells. CMV is therefore considered to be a particular pathogen to which the host immune system pays close attention.

#### *Immunosuppressive agents increasing the risk of CMV infection*

Immunosuppressive agents are apparently the most important exogenous factor of CMV infection in transplant recipients. Conventional immunosuppression was achieved using azathioprine and prednisolone in the 1970s, cyclosporine-based therapy in the 1980s into the 1990s, and FK506-based therapy in the 1990s. In some cases, anti-lymphocyte antibody had to be added to the conventional immunosuppression regimen to prevent ongoing rejection episodes.

Anti-lymphocyte antibodies including such polyclonal antibody as anti-lymphocyte globulin (ALG) and anti-thymocyte serum (ALS), and such mAb as OKT3 have been identified as the risk factors for the development of CMV disease<sup>6, 7</sup>. Various clinical results have

been reported as for the roles of other chemical immunosuppressants, including purine analogues (azathioprine, mycophenolate mofetil), calcineurin inhibitors (cyclosporine, FK506), and rapamycin, in the development of CMV disease. KUYPERS et al.<sup>12</sup> reported the risks of various immunosuppressive drugs and combined drug regimens. In the study, the combined use of FK506, mycophenolate mofetil (MMF) and steroids (SFM protocol) was associated with CMV disease, although SFA (FK506+azathioprine+steroids) was not. Thus, it was possible that MMF is a risk factor for CMV disease. Actually, some trials indicated that MMF as a risk factor CMV infection<sup>3, 4, 26</sup>, while another group could not confirm this result<sup>18</sup>.

Why does OKT3 increase the incidence of infectious complications? As mentioned above, a high frequency of CMV-specific T cells is required as "guardian" cells presumably in order to inhibit CMV (re)activation. The depletion of whole T cells, including CMV-specific T cells, may allow CMV (re)activation and, consequently, trigger CMV-associated disease. Indeed, it has been reported that 0.25% of CD4<sup>+</sup> CMV-specific T cells was the critical point of subsequent CMV disease<sup>19</sup>. Although it is true that OKT3 is a useful material for both controlling and preventing a rejection episode, this mAb may deplete T cells so vigorously that it allows CMV reactivation. To compensate this demerit, mAbs recognizing various phenotypic markers on T cells have been developed. Among them, a mAb recognizing CD25 (interleukin-2 receptor – IL-2R) has demonstrated its therapeutic efficacy. We previously reported that ART-18 (mouse anti-rat IL-2R mAb) prolonged cardiac allograft survival by eliminating IL-2R-positive cells<sup>21, 25</sup>. Similar results have been reported from various laboratories. However, recipient immune response against the xenogenic mAb has limited its clinical use<sup>9</sup>. This disadvantage has been overcome by the development of a chimeric (mouse/human) mAb in which only portions of the CD25 binding site are derived from an original rodent antibody, while the remaining portions are human in origin. In a multi-center clinical trial, this material has been shown to reduce the occurrence of acute allograft without increasing the risk of CMV infection<sup>13</sup>. This mAb would spare CMV-specific memory/effector T cells.

### Concluding Remarks

Recent technology has allowed us to measure virus-specific T cells. The results obtained from experiments

using the new tools have revealed various types of evidences in the field of viral immunology. As for CMV, a high frequency of specific T cells has recently been shown to be required presumably to survey CMV reactivation. In this review, the relationship of immunosuppressive agents and CMV infection was discussed based on new findings obtained from experiments measuring CMV-specific T cells. In addition, the CMV genome number can now be measured with a highly sensitive method using real time polymerase chain reaction<sup>13</sup>. At the time of this review, many investigators, including ourselves, are continuing their efforts to accumulate results by measuring specific T cells and CMV genomes in human materials and mouse models. These results will hopefully allow us to identify improved immunosuppressive agents for use in transplant recipients to prevent CMV infection more effectively.

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