SUMMARY

This dissertation is based on clinical investigations of the diagnosis, management, and immunological aspects of cytomegalovirus (CMV) infections in renal and liver transplant patients.

CMV is a member of the herpes virus family, that also comprises varicella-zoster virus, herpes simplex virus, and Ebstein-Barr virus. About 50% of all adults have experienced infection with CMV, generally during infancy; the majority of these infections being asymptomatic. CMV is not eliminated completely from the body after infection, but remains present in a latent form. This latency can be recognized by the presence of antibodies against CMV. Although the molecular basis of CMV latency is far from understood, it is evident that antiviral immune responses are necessary to maintain this state. In case of impaired function of the immune system (e.g., due to immunosuppressive drugs, or to infection with the human immunodeficiency virus) CMV may reactivate and cause disease.
CMV infection occurs in 50-70% of all transplant recipients, and may be the result of reactivation of the endogeneous viral strain, or of transmission of the virus via the graft. Fortunately, many infections remain asymptomatic because the immune system is still sufficiently intact to mount rapid antiviral responses and limit the degree of viral replication. CMV disease occurs predominantly when there has been no previous exposure to CMV (and therefore no immunological memory exists that can be rapidly recruited), or when the capacity to respond has been severely reduced by intensive antirejection treatment. Clinical manifestations of CMV disease consist of high, often spiking fevers, malaise and arthralgias, and may be accompanied by symptoms and signs of organ involvement, especially esophagitis, gastritis, hepatitis or pneumonitis.
There are indications that CMV indirectly causes illness by exerting an immunosuppressive effect that increases the susceptibility to other opportunistic agents.

The immune system plays a pivotal role with respect to the outcome of organ transplantation. Therefore, chapter 1 first provides a short summary of the function of this system, describing the way by which foreign antigens are recognized and eliminated. This functioning is complicated in organ transplant recipients by the immunosuppressive drugs prescribed in order to prevent rejection of the graft. These agents non-specifically suppress immune reactivity, including responses against infectious organisms, resulting in a greatly increased risk of opportunistic infections. CMV is the most frequent cause of infections in transplant patients, and the remainder of chapter 1 describes the virological, clinical and immunological aspects of CMV infection. Two recent developments in this field form the basis of this thesis: the CMV antigenemia assay, a sensitive and

quantitative method for early diagnosis and monitoring of infection; and the availability of ganciclovir, a nucleoside analog that effectively suppresses viral replication and provides the first specific therapeutic option against CMV.

Chapter 2 offers a working hypothesis for the relation between CMV and the host immune system. This relation can be envisaged as a bilateral interaction: on the one hand, the immune system suppresses viral replication. On the other, CMV negatively influences host immunocompetence. During health a balance exists, with CMV subclinically just active enough to stimulate low production of antibodies, yet otherwise kept in latency by the immune system. This balance is disturbed by immunosuppressive treatment, with CMV disease as a potential consequence. The viral replication during disease forms a stimulus for the immune system to restore the initial balance. Treatment with antiviral drugs only temporarily restores the balance, and the ultimate outcome depends on whether antiviral immune responses have developed and are able to consolidate the treatment result.
The main questions of this thesis follow directly from the working hypothesis: What is the value of CMV antigenemia as a diagnostic marker for CMV infection? What is the importance of the viral load for the development of CMV disease? What are the effects of antiviral therapy on clinical symptoms and on the viral load? What are the clinical and immunological consequences of CMV infection for the immuune status of the patient? Can early intervention with antiviral drugs limit the negative effect of CMV on the immune system?

Chapter 3.1 describes a comparison of the CMV antigenemia assay, viral isolation from blood and urine, and serology for the early diagnosis of CMV infection in 72 renal transplant patients. Antigenemia and serology proved to be the most sensitive methods to detect infection, but viral isolation performed equally well in diagnosing CMV disease. Antigenemia could be detected 8 days before isolation of virus from blood, and 11 days before onset of an antibody response against CMV.
Detection of viral DNA in clinical samples using the polymerase chain reaction (PCR) represents a revolutionary development in the field of diagnostic virology. In an analysis of 103 blood samples, PCR (89%) and the antigenemia assay (86%) proved to be more sensitive than rapid viral isolation (45%).
These data indicate that the antigenemia assay and PCR are superior to rapid viral isolation and serology with respect to early diagnosis of CMV infection after organ transplantation.

Chapter 3.2 describes a study of the value of weekly testing of CMV antigenemia for the early detection and monitoring of CMV disease in a group of 130 renal transplant patients, 22 of which developed a CMV syndrome. In 19 (85%) of them, antigenemia was present already before, or at the onset of, clinical symptoms. The pattern of antigenemia during infection correlated well with symptomatology. The maximum number of antigen-positive cells during infection was higher, and the duration of antigenemia was longer, in patients with mild or severe CMV disease than in those with asymptomatic

infection. The base
appearance of antig
CMV disease led t
of CMV syndrome.
intervention strateg

Differentiation of a
chapter 3.3 we ana
antigenemia assay i
Absence of antigen
dysfunction (of cou
Eleven of the 39 re
number of antigen-
the 10 patients witl
< 10 positive cells
rejection) this num
If carefully interpr
of these "difficult"

Chapter 4.1 descril
symptomatology ar
Eight of 11 patient
dropped concurren
illness despite anti
days of treatment.
Relapse of CMV d
rises of antigenemi
remained below th
complications (reje
antiviral treatment

Simultaneous sterc
dilemma. Treatme
severe CMV disea
certainly lead to g
with this unhappp
instituted. This le
disease occurred.
stopped. Antigene
as for monitoring

Chapter 4.3 sumn
emptive treatment

infection. The baseline risk of a CMV syndrome was 19%, and rose to 42% upon appearance of antigenemia. Combining antigen-positivity with well-known risk factors for CMV disease led to the delineation of subgroups with significantly higher risk (60-86%) of CMV syndrome. These categories are relevant for the development of early intervention strategies.

Differentiation of allograft rejection from CMV disease may be a difficult exercise. In chapter 3.3 we analyzed in a group of 73 renal transplant patients whether the antigenemia assay is of additional value in this situation.
Absence of antigenemia practically excluded CMV as the cause of fever and allograft dysfunction (of course, this does not prove that the abnormalities are due to rejection). Eleven of the 39 rejection episodes occurred in the presence of CMV antigenemia. The number of antigen-positive cells was helpful in diagnosing CMV disease in these cases: in the 10 patients with asymptomatic infection (and concurrent rejection), this number was <10 positive cells per 50,000 PMN's whereas in the patients with CMV disease (and rejection) this number ranged between 11 and 99.
If carefully interpreted, the antigenemia assay can be helpful in guiding the management of these "difficult" cases.

Chapter 4.1 describes the effects of antiviral treatment with ganciclovir on clinical symptomatology and antigenemia levels in patients with severe CMV disease.
Eight of 11 patients rapidly improved under ganciclovir treatment; in 7/8, antigenemia dropped concurrently with clinical improvement. Three patients had prolonged CMV illness despite antiviral therapy. In these 3 antigenemia levels increased during the first days of treatment.
Relapse of CMV disease after discontinuation of ganciclovir was associated with renewed rises of antigenemia levels beyond 10 positive cells per 50,000 PMN's; antigenemia remained below this level in patients with an uncomplicated recovery, or with other complications (rejection, other infections). Viral isolation was generally negative during antiviral treatment regardless of the clinical response.

Simultaneous steroid-resistant rejection and CMV infection forms a difficult therapeutic dilemma. Treatment with anti-thymocyte globulin (ATG) or OKT3 may well lead to severe CMV disease, but failure to administer intensive antirejection therapy will almost certainly lead to graft loss. Chapter 4.2 presents a solution to this dilemma. In 4 patients with this unhapppy combination, combined treatment for rejection and CMV infection was instituted. This led to recovery of renal function, while mild or no symptoms of CMV disease occurred. Two of the 4 patients had recurrent CMV disease after ganciclovir was stopped. Antigenemia testing proved useful, both for early recognition of infection as well as for monitoring the effects of treatment.

Chapter 4.3 summarizes the results of a pilot study of the feasibility and efficacy of pre-emptive treatment of primary CMV infection. CMV syndromes occur in over 70% of all

patients with primary infection: can disease be prevented by low-dose ganciclovir, started when the patient still is asymptomatic?
Nine CMV seronegative patients were randomized upon appearance of antigenemia: 5 were treated expectantly, and 4 received ganciclovir at 50% of the usual therapeutic dosage for 10 days. All 5 controls developed CMV disease within a few days after randomization, but none of the patients on ganciclovir. However, after discontinuation of ganciclovir 3 of the 4 preemptively treated patients had one or more episode(s) of CMV disease, accompanied by exceptionally high viral loads. This may have been caused by antirejection treatment, or may reflect a negative effect of antiviral therapy on the development of antiviral immune responses.

There are indications that CMV infection negatively affects host immunocompetence. In chapter 5.1 we investigated whether this is mediated by lymphocyte dusfunction.
Proliferation of lymphocytes in vitro in response to stimulation with immobilized αCD3 was significantly reduced in patients with CMV infection. Co-stimulation experiments using αCD28 indicated that this pathway probably is intact.
The decreased proliferation rate was not just the result of a relative increase of activated, poorly proliferating CD8+ T cells.
During CMV infection a median of 15% (range, 6-60) of all peripheral blood lymphocytes were undergoing apoptosis (programmed cell death), whereas this number was always lower than 5% in patients without CMV infection. The degree of apoptosis was independent of the viral load. CMV-related apoptosis might be the result of defective T-cell activation by CMV-infected antigen-presenting cells.

What are the clinical consequences of these in vitro cellular abnormalities? This question forms the main topic of chapter 5.2, which contains an analysis of infectious complications after orthotopic liver transplantation in a group of 111 consecutive patients. The incidence of major infectious complications (other than CMV) during the first 30 days after transplantation was comparable in patients with and without (subsequent) CMV infection. After that time, patients with CMV infection developed other major infections significantly more often than those who did not have CMV infection. Interestingly, the majority of these other major infections were caused by bacteria. This suggests that the increased susceptibility to infections was caused by decreased barrier function of the skin or mucosal surfaces, or impaired phagocytic function. Relatively few infections were caused by fungi, yeasts, and protozoa (the principal opportunistic agents in patients with abnormal T cell function), and their incidence was comparable in patients with and without CMV infection. This finding may reflect the beneficial effect of timely reduction of immunosuppression during CMV disease that prevented an increase in the number of these life-threatening infections.

Chapter 6.1 describes the development of an ELISA system for the quantitative measurement of IgM- and IgG antibodies against CMV. Using this method the levels of antibodies during primary and secondary CMV infections after renal transplantation were studied. Especially during secondary infections a rapid IgG response against CMV

appeared to prevei

Cellular immune r
Monitoring these
implementation. W
lymphocytes (that
marker of cellular
renal transplant pa
During the recove
activated CD8^{bright}
(natural killer cell
activated CD8^{bright}
severe, progressiv
of peripheral bloo
against CMV. Mo
the recovery from
antibodies against
load.

Cellular immune a
e.g., IL-2R, CD4
these soluble prod
CMV.
Baseline levels of
infection, whereas
sCD8 showed the
infection. Howeve
progressive diseas
guarantee for effe
Levels of sCD8 a
However, in patie
numbers of activa
result from an inc

Chapter 5.6 conta
patients with CM
from 6% before i
exceeded the upp
degree of activati
These data are co

As previously dis
treatment form a

appeared to prevent CMV disease.

Cellular immune responses probably form the cornerstone of the defense against CMV. Monitoring these responses is technically difficult and too inconvenient for routine implementation. We hypothesized that the activation status of peripheral blood lymphocytes (that can be easily measured using flow cytometry) might form a (surrogate) marker of cellular immune responses against CMV, and investigated this hypothesis in renal transplant patients with primary and secondary CMV infection (chapter 6.2). During the recovery phase of uncomplicated primary CMV infection the number of activated $CD8^{bright}$ lymphocytes (cytotoxic T cells) and activated $CD56^{+}$ lymphocytes (natural killer cells) strongly increased. During recovery from secondary infection, only activated $CD8^{bright}$ cells increased. Numbers of these activated cells remained lowduring severe, progressive primary CMV infection. These data suggest that the activation status of peripheral blood lymphocytes forms a surrogate marker of cellular immune responses against CMV. Moreover, cellular and humoral immunity appear to have different roles in the recovery from CMV infection: cellular responses interrupt viral replication, while antibodies against CMV may give symptomatic improvement without changing the viral load.

Cellular immune activation is associated with the release of several membrane molecules, e.g., IL-2R, CD4, CD8 and CD27. We evaluated in chapter 6.3 whether monitoring these soluble products also forms a useful marker of cellular immune reactivity against CMV.
Baseline levels of sCD4 were low, and remained within the range of normal during CMV infection, whereas the other 3 markers significantly rose during CMV infection. Rises of sCD8 showed the strongest increase, and occurred especially during recovery from infection. However, in several patients sCD8 (and the other markers) rose also during progressive disease, indicating that activation of cytotoxic T cells is in itself not a guarantee for effective antiviral effector responses.
Levels of sCD8 and numbers of activated $CD8^{bright}$ cells were strongly correlated. However, in patients with progressive disease sCD8 often rose disproportionately high to numbers of activated cells compared with patients with self-limited infection. This might result from an increased rate of cell death of $CD8^{bright}$ cells during progressive disease.

Chapter 5.6 contains an analysis of natural killer (NK) cell function in 15 renal transplant patients with CMV infection. NK activity, as measured in a K562 lysis assay, increased from 6% before infection to a peak value of 25 % during infection ($P < .001$), and exceeded the upper level of controls in 9/15 cases. NK function was correlated with the degree of activation of these cells (as measured by flow cytometry) ($r = .57$, $P < .001$). These data are consistent with a role for NK cells in recovery from CMV infection.

As previously discussed, relapses of CMV disease after initially successful antiviral treatment form a clinically relevant problem. We retrospectively analyzed in a group of

36 renal- and liver transplant patients whether these relapses can be predicted. Clinical characteristics (CMV serostatus of donor and recipient, type of immunosuppression, antirejection therapy, indication for antivirals) and virological parameters (antigenemia levels before, during, or at the end of treatment, PCR positivity after treatment) were not helpful in predicting subsequent relapse. However, antigenemia levels rose strongly at the time of recurring disease: 10 or more antigen-positive cells per 50,000 PMN's occurred in 10/11 patients with, and 1/25 without relapse ($P < .001$). Humoral immune responses at completion of treatment were of no predictive value in the 15 patients with primary infection, but absence of an IgG response in those with secondary infection may be an indicator of impending relapse.
Numbers of activated $CD8^{bright}$ and $CD56^+$ lymphocytes at the end of antiviral treatment were available from 15 patients. None of 8 patients with more than $100x10^3$/ml $HLADR^+$ $CD8^{bright}$ cells had recurrent disease, whereas 6 of 7 patients with a lower number relapsed ($P < .01$). No evident prognostic value of numbers of activated $CD56^+$ cells was found in this small group of patients. These data suggest that specific cellular immune responses determine the ultimate outcome of antiviral therapy.

Chapter 6 summarizes and integrates the results from the previous chapters. It is concluded that viral load forms the most important determinant of CMV disease in this category of immunosuppressed patients. The viral load is, in its turn, regulated by host immune responses and antiviral therapy.
Not withstanding that many aspects of the immunopathogenesis of CMV disease, as well as the specificity and regulation of immune responses are poorly understood, specific guidelines for the management of transplant patients with CMV infection or CMV disease are available.
This thesis concludes with some suggestions for further study.

Dit proefschrift b
diagnostiek, beha
infekties bij nier-