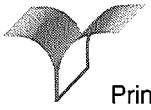
DIAGNOSIS AND PREVENTION OF CYTOMEGALOVIRUS INFECTION AFTER ORGAN TRANSPLANTATION



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DIAGNOSIS AND PREVENTION OF CYTOMEGALOVIRUS INFECTION AFTER ORGAN TRANSPLANTATION

Diagnostiek en preventie van Cytomegalievirus infectie
na orgaantransplantatie

PROEFSCHRIFT

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CONTENTS

PROMOTIECOMMISSIE.

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CHAPTER 1

INTRODUCTION

After the first description of a generalized Cytomegalovirus (CMV) infection in a kidney transplant recipient by Rifkind et al in 1964 [1], it became clear that in these patients the incidence of this infection was high, as summarized by Betts in 1982 [2]. Although a majority of the infections may run an asymptomatic course, in some patients CMV infection is associated with morbidity and to a lesser extent mortality. It seems that the introduction of cyclosporin A (CsA) as the main immunosuppressive agent in organ transplantation has affected the incidence and severity of CMV infection in a favourable way. Nevertheless, even under CsA therapy the reported incidence of CMV infection ranges from 37 - 72 % and of clinical overt CMV disease from 2 - 23 % [3 - 9]. In recipients of other organs, e.g. heart and liver, the incidence of CMV disease is reported to be even higher, probably as a result of the more vigorous immunosuppressive therapy used in these patients [10 - 14]. It is therefore obvious that CMV remains a major pathogen after organ transplantation. In this chapter a general introduction is given on CMV infection, both in the immunocompetent host and in the immunosuppressed allograft recipient.

EPIDEMIOLOGY

In immunocompetent individuals primary CMV infection induces cellular and humoral immune responses leading to immunity as reflected by seropositivity. According to the serostatus of the population CMV infection is endemic rather than epidemic and it occurs throughout the year rather than being seasonal. In the majority of the populations studied, the age at which CMV antibody is acquired is dependent on race, socioeconomic conditions and sex [2]. Cytomegaloviruses are ubiquitous agents with a cumulative exposure rate of 70 to 80 %, as suggested by epidemiological studies in the United States, England and Sweden [15]. Between 0.5 and 2.2 % of newborn babies shows signs of in utero infection [16]. Another 8 to 60 % becomes infected during the first 6 months of life, as a result of transmission of the virus during birth [16, 17], through breast feeding [18] or from close contact with other children [19 - 21]. In most developed countries infection rates increase slowly after infancy until the age of schoolentry, at which time they rise more rapidly; 40 to 80 % of children are infected by the time they reach puberty [22]. Oral and respiratory spread appears to be the dominant route of transmission during childhood. In adulthood CMV can be spread through venereal route too, as is suggested by the observation that infection rates, as assessed by antibody status, are much increased in promiscuous populations [22]. Bloodproducts and organ transplants have also been implicated as source of CMV [23, 24].

PATHOLOGY AND PATHOGENESIS

Human CMV is a virus with low pathogenicity in immunocompetent individuals. CMV is cytopathic in vivo as manifested by the CMV inclusion bodies. In vitro, CMV, when inoculated into permissive human fibroblasts, produces a characteristic cytopathic effect within hours or weeks following inoculation. This cytopathic effect is characterized by the appearance of large refractile cells occurring focally. These cells contain intranuclear inclusions identical to those observed in vivo [22]. CMV infections run in most immunocompetent individuals a subclinical course. In sporadic cases CMV is known to cause a mononucleosis like syndrome [25, 26]. This disease is self-limiting, but may be complicated by interstitial pneumonia [27], hepatitis [28, 29], Guillain-Barré syndrome [30 - 32], meningoencephalitis [30, 33], polyarteritis nodosa [34], myocarditis, thrombocytopenia and hemolytic anemia [15, 35]. More recently CMV has been associated with atherosclerosis [36, 37], malignancy [38] and erythema nodosum [39].
As with other human herpesviruses, primary CMV infection is followed by persistence of the virus (or at least the viral genome) in the host for a long, maybe lifelong period. CMV persistently infects epithelial cells of the salivary gland and the glomerulus and tubulus of the kidney [40, 41]. In addition, CMV may persist in lymphocytes, neutrophilic granulocytes [42, 43] and vascular smooth-muscle cells [36]. Epidemiologic studies suggest that CMV may persist in other organs too, as CMV can be transmitted with the donated heart [10], liver [44] or skin [45] during organ transplantation.

Reinfection may occur in immunocompetent persons because of the antigenic and genetic diversity among CMV strains [46 - 48].

IMMUNOLOGY

A primary CMV infection is followed by a humoral and cellular immune response in immunocompetent individuals. IgM antibodies peak during the early course of infection and disappear 12 to 16 weeks after the onset of (sub)clinical infection [49]. IgG antibodies peak during the first 4 to 6 weeks after onset of infection [26]. The IgG antibody response appears to be restricted [50]. IgG1 and IgG3 are produced in primary infection and IgG1 is the main responding subclass in reactivated disease. Moreover, IgA is produced during primary and reactivated CMV infection [50] and IgE is only produced during primary infection [51]. The antibody response is directed against various CMV antigens. At least 30 virus-encoded proteins have been described in the virion and more than 50 in infected cells [52]. The sequence of protein production after CMV infection of cells reveals an interdependent cascade of immediate early, early, and late protein production [53]. Most of the proteins are internal structural proteins and are probably not expressed on the viral envelope or the membrane of infected cells. So far only 20 proteins appear to be immunogenic in humans and evoke antibody responses in vivo [52]. However, not all of the antibodies to these proteins might play a role in the host defense against CMV.

Cell-mediated immunity develops after the appearance of the specific viral antibodies. CMV-induced lymphocyte reactivity of peripheral blood lymphocytes is reported to appear more than 50 days after onset of infection [54]. In the early period

of CMV infection cellular immunity is rather depressed than enhanced, as is shown by the hyporesponsiveness of mononuclear lymphocytes to certain mitogens (pokeweed and concanavalin A) in patients with a CMV mononucleosis [55 - 57]. In contrast, the proliferative responses of mononuclear cells to phytohemagglutinin and allogeneic lymphocytes are normal in immunocompetent patients during CMV infection [56]. This change of the lymphocyte proliferative responses to T- and B-cell mitogens lasts for about four to five weeks after the onset of infection. During acute CMV infection a reversal in the ratio of CD 4-positive and CD 8-positive cells is found. This reversal is caused by an absolute increase in CD 8-positive cells and a slight decrease in CD 4-positive cells. The atypical lymphocytes that characterize the mononucleosis appear to be activated CD 8-positive cells [58 - 61]. This suggests that the mitogen hyporesponsiveness in CMV mononucleosis may be mediated by an increase in CD 8-positive cells. Moreover, CMV can induce a polyclonal activation of B lymphocytes [62]. This may explain some of the serologic abnormalities found during CMV infection, as e.g. the appearance of mixed cryoglobulins, cold agglutinins, rheumatoid factors and antibodies against smooth muscle [63].

Although cellular immunity is depressed during the early period of infection, it is nevertheless the main defence against CMV infection [64]. This antiviral cytotoxicity may be either T-cell or non T-cell mediated. The non T-cell lymphocytes have the characteristics of natural killer cells and antibody dependent killer cells [65 - 68].

The T-cell component of this cytotoxicity subsides after resolution of the acute infection, since CMV-specific cytotoxic cells in the peripheral blood of non-immunocompromised persons are non-T lymphocytes [69]. Depression of this cell-mediated immunity, for instance induced by treatment with immunosuppressive drugs in allograft recipients, causes a high incidence of symptomatic CMV infection.

CMV INFECTION AND ORGAN TRANSPLANTATION

INCIDENCE

The first 3 to 4 months after allograft transplantation is the period of greatest risk of developing symptomatic CMV infection [2, 70, 71] However, CMV-excretion can persists for 2 - 14 years after transplantation and is sometimes associated with clinical illness during that episode [72]. The incidence of CMV infection is high after renal transplantation. Table 1-1 shows the results of 8 studies including data from 923 patients, who were treated with AZA as the main immunosuppressive agent. The incidence of CMV infection ranged from 48 - 89 %, and symptomatic disease was reported in 17 - 25 %. It seems that the introduction of CsA as the main immunosuppressive agent in organ transplantation has affected the incidence and severity of CMV infection. Most studies suggested a lower incidence in CsA-treated compared to azathioprine-treated patients [6, 7, 77], although this was not confirmed by others [4, 5]. Under CsA therapy the reported incidences of CMV infection ranged from 37 to 72 %, while clinical overt CMV disease ranged from 2 to 23 % (Table 1-2).

Reference Number	Author	Number of patients	Percentage with CMV infection	Percentage with CMV disease
3.	Peterson et al.	51	n.s.	22
4.	Harris et al.	212	82	n.s.
5.	Bia et al.	40	48	21
73.	Andrus et al.	120	73	n.s.
74.	Marker et al.	320	53	n.s.
75	Flechner et al.	100	58	23
76.	Gadler et al.	28	89	25
77.	Luby et al.	52	85	17

Table 1-1 Reported incidence of CMV infection and disease in renal transplant recipients treated with azathioprine and prednisone in eight studies. n.s. Not stated.

Reference Number	Author	Number of patients	Percentage with CMV infection	Percentage with CMV disease
3.	Peterson et al.	48	n.s.	2
4.	Harris et al.	61	72	n.s.
5.	Bia et al.	24	58	22
6.	Najarian et al.	121	n.s.	9
7.	Can. Multicentre Tr. Study Group	142	n.s.	4
8.	Weir et al.	162	n.s.	9
9.	Johnson et al.	376	37	23

Table 1-2 Reported incidence of CMV infection and disease in renal transplant recipients treated with cyclosporine and prednisone in seven studies. n.s. Not stated.

CLINICAL SYMPTOMS

Fever is the most prominent symptom of CMV infection. It is typically episodic with morning spikes of 40° C or more. These spells can continue for three to four weeks and are frequently accompanied by arthralgias only. Other symptoms related to CMV infection include rigors, fatigue, anorexia, abdominal pain and diarrhoea [70]. Leucocytopenia and thrombocytopenia can be present too. CMV has also been related to hepatic damage [78]. Abnormal liver function tests are diagnosed in up to 45 % of the infected patients [79]. Bleeding from the gastrointestinal tract occurs in 20 % of the symptomatic patients. Most of these complications are caused by ulcerative lesions in esophagus, stomach, duodenum and colon secondary to vasculitis [80 - 82]. Pancreatitis is seen in a smaller group of patients and pneumatosis intestinalis is a rare complication related to CMV infection in organ transplant recipient [83]. Other less frequently observed complications of CMV infection after organ transplantation are thyroiditis [84], myelitis [85], pericarditis, myocarditis and retinitis [70, 87].

The most feared complication of CMV infection after organ transplantation is interstitial pneumonitis. The clinical picture in patients with CMV pneumonitis can vary between a decreased diffusing capacity for carbon monoxide without radiologic abnormalities or abnormal bloodgases and a fulminant diffuse pneumonitis with a high fatality rate [87, 88]. The pathogenesis of CMV interstitial pneumonitis is not well understood. Clinical observations and studies in animal models have suggested that an immunopathological reaction rather than ongoing virus replication and its cytopathic effect are responsible for the severity of the pneumonitis. In patients receiving allogeneic bone marrow, graft- versus host (GvH) disease is often associated with the development of CMV pneumonitis [89]. DHPG (Ganciclovir) given to bone-marrow transplant recipients with CMV pneumonitis reduced the CMV titer in the lungs by more than 99.9 %, but 9/10 treated patients died from their pneumonitis, which suggests a poor correlation between titers of virus in the lung and the severity of pneumonitis in these patients [90].

In the nude mouse model MCMV replicates extensively in the lung, but diffuse pneumonitis does not develop until the terminal stage of infection. When T-cell function is reconstituted with syngeneic cells before the mice are challenged with MCMV, pneumonitis did develop. Thus, some component of the T-cell immune response and not virus replication per se is important in the induction of pneumonitis by MCMV in this model [91, 92].

CMV AND RENAL ALLOGRAFT SURVIVAL

The influence of CMV infection on renal allograft survival is still a controversial issue. Infection with CMV has been associated with allograft rejection and poor graft survival [70, 93 - 99]. However, in most of these studies it could not be determined whether infection activates allograft rejection or rejection and anti-rejection therapy (re-) activates a virus infection. Moreover, other authors found no correlation between rejection episodes and CMV infections [100 - 105].

There are some explanations for this supposed detrimental effect of CMV infection on renal allograft survival. Firstly, CMV infection can induce expression of

class I and class II HLA-antigens and elicit rejection [106, 107]. Secondly, it is suggested that CMV infection diminishes the positive effect of bloodtransfusions on allograft survival [108]. Thirdly, CMV infection can induce glomerulopathy. These glomerular lesions can be managed by a decrease in immunosuppressant therapy [109, 110], although these observations could not be confirmed by others [111, 112].

RISK FACTORS FOR CMV INFECTION

Many factors can influence the incidence and severity of CMV infection after organ transplantation. Primary infection runs a more serious course after transplantation than secondary infection [2]. In most cases the transplanted organ is the source of CMV, as was shown independently in 1975 by Ho et al. and Betts et al. [113, 114]. After these two first publications a number of epidemiological studies (mostly from the U.S.A and the U.K.) concerning the potential risk of transferring CMV through allografts have been published. Table 1-3 shows the pooled data of 6 studies in 205 CMV seronegative renal allograft recipients and the influence of the CMV serostatus of the donor and the acquisition of CMV infection. The incidence of CMV infection was 6 times higher in recipients of a kidney from a CMV seropositive donor as compared to those of a kidney from a CMV seronegative donor. A majority of these infections were symptomatic.

CMV serostatus Renal allograft Donor/Recipient	Number of combinations	Percentage with CMV Infection	Percentage with CMV disease
POS/NEG	193	69	41
NEG/NEG	112	11	0

Table 1-3 **Reported incidence of CMV infection and disease in two groups of CMV seronegative renal transplant recipients, according to the CMV serostatus of the allograft donor. Pooled data of 6 studies (ref.: 4, 94, 103, 113 - 115).**

More recently, it was shown that CMV transmission through allografts is also possible in CMV seropositive recipients [116 - 118]. Other allografts, as heart, liver and skin can transfer CMV too [10 -14]. Blood products have also been implicated as a source of CMV. The relative risk happened to be proportional to the number of units transfused [119]. The overall risk has been calculated to be 12 - 24 seroconversions / 100 units blood transfused in immunocompetent recipients [23]. Moreover, it was shown that 7/223 (3 %) volunteer blood donors were shedding CMV in the urine [120]. Leucocytes in the blood are the most likely source of CMV, as was shown by in situ hybridization studies [43]. Another indirect proof for this comes from

the observation that leucocyte - depleted blood prevents CMV infection in neonates [121]. In renal allograft recipients no clear relationship between the incidence of CMV infection and the number of bloodunits received during or after a transplantation has been shown so far [122 - 125]. However, in none of these studies all the variables such as CMV serostatus of recipients, kidney and blooddonors were monitored.

Another important risk factor for acquiring symptomatic CMV infection is the immune status of the host. Type and amount of immunosuppression are important factors. Both high doses of corticosteroids and the use of antilymphocyte globulins, including anti T - cell monoclonal antibodies, have been associated with a higher incidence and severity of CMV infection in renal transplant recipients [71, 126 - 128] However, when the dose of azathioprine and prednisone administered to patients receiving ALG was reduced by approximately 50 % there was no increase in the incidence of CMV viremia or related disease [129]. This suggests that the net state of immunosuppression rather than the individual components of the immunosuppressive regimens is responsible for CMV infection. This is in accordance with the observation by Hoitsma et al [130]. They reported no difference in incidence of CMV infection, whether renal transplant recipients were treated with rabbit antithymocyte globulin or with steroids for rejection.

DIAGNOSIS OF CMV INFECTION

The diagnosis of CMV infection is based on isolation of virus in cell culture or detection of virus-specific antibodies. Both methods have disadvantages. Serological techniques such as immunofluorescence (IF) and enzyme linked immunosorbent assays (ELISA) may reveal false-positive or false-negative results and tissue culture methods are cumbersome, time consuming and sometimes impossible due to microbacterially contaminated specimens or coinfection with the herpes simplex virus. Some new advances have recently been made in rapid viral diagnosis. A hybridization assay for detection of CMV DNA in clinical specimens has been shown to be a highly specific and rapid diagnostic procedure [131, 132]. Furthermore centrifugation in combination with an immunofluorescence assay speeded up the diagnosis of CMV infection to 24 to 48 hours after inoculation [133]. More recently, an immunocytologic assay for the detection of CMV immediate early antigen in circulating blood leucocytes was described [134].

More indirect diagnosis for viral infection comes from the monitoring of peripheral blood mononuclear subpopulations with monoclonal antibodies [135]. Renal allograft recipients with azathioprine as basic immunosuppression showed an inversion of the CD4/CD8 ratio (< 1.0) during CMV infection [136]. However, in patients on cyclosporin A this phenomenon is thought to be less evident [110, 135 - 138].

PREVENTION AND THERAPY OF CMV INFECTION

Apparent avenues to deal with viral infections are antiviral chemotherapy, immunization, stimulation of nonspecific humoral and cellular mechanisms and avoidance of viral transmission. All have been tried in an attempt to control the CMV

infections. Until recently, results of treatment with antiviral agents have been disappointing. Treatment with vidarabine in allograft recipients have been uniformly unsuccessful in disseminated disease [139], although in incidental patients successful treatment with vidarabine of CMV encephalitis has been reported [33]. Acyclovir in high concentrations has in vitro activity against CMV [140]. However, both prophylaxis and treatment of CMV infection with acyclovir have been unsuccessful in allograft recipients [141 - 145], although some studies have observed protection against CMV infection in subgroups of bone-marrow and renal transplant recipients [146 - 148]. Especially in CMV seronegative recipients of a CMV seropositive renal allograft high-dose (800 - 3200 mg per day) acyclovir has been reported to prevent CMV infection and disease, although this study has to be confirmed.

The therapeutic efficacy of a new guanine-analogue, 9-(1,3 dihydroxy 2 propoxymethyl) guanine (DHPG, Cymevene) seems more promising [149], although the therapeutic efficacy in CMV pneumonia was still absent in the patients with HIV - infection as underlying disease. In organ transplant recipients with CMV pneumonitis the results of treatment with DHPG are better [150 - 156]. However, all these studies have been uncontrolled and the results of DHPG treatment in patients with encephalitis and pneumonitis have not been uniformly successful. Moreover, recovery of CMV strains resistant to DHPG from the blood of patients treated with DHPG are reported [157]. Another antiviral agent, foscarnet, has been reported to be effective in transplant patients with a CMV pneumonia [158, 159] and is now advocated as a rescue drug in DHPG resistant CMV infections [160].

Interferon (IFN) alpha en beta has been used both therapeutical [161] and prophylactically [72, 162 - 165] in CMV infection after renal allograft transplantation. In two studies [163, 164] a significantly reduction of CMV disease in the interferon group was found. However, induction of steroid resistant rejections were observed in renal transplant recipients treated with recombinant IFNα 2 [165, 166]. Therefore the benefit derived from the lower incidence of CMV infection in interferon treated patients was nullified by the occurrence of severe rejections.

Based on the observations that primary CMV infection follows a more severe course than secondary infection and cellular immunity plays a much larger role in prevention and combatting of CMV infection than humoral immunity, active immunization seems to be a logical strategy to control CMV infection after organ transplantation. Some trials of active immunization using live attenuated strain of CMV have been conducted [167, 168]. Although it was shown that the Towne vaccine is safe and immunogenic in renal transplant recipients it did not prevent CMV disease in all cases. Especially CMV seronegative recipients of a CMV seropositive allograft developed in a high incidence CMV disease [167]. However, primary infection seemed to follow a milder course.

Another option to prevent CMV infection after organ transplantation is passive immunization with anti-CMV immunoglobulin preparations. It has been shown in animal studies that serum derived from mice within three to five days of CMV infection contained a complement - dependent neutralizing antibody of the IgG type which, when passively transferred, protected mice from a subsequent lethal infection challenge with CMV [169]. Other studies in animal models could confirm this observation [170]. In humans, it has been demonstrated that congenital CMV infections occur almost exclusively in infants born to woman primarily infected with CMV during pregnancy [171]. Women who experienced reactivation of CMV during pregnancy gave birth to normal offspring. However, some of them showed a CMV viruria after birth without clinical symptoms. The protection given to neonates of

secondary infected mothers appeared to be based exclusively on transplacental passage of maternal antibody of the IgG class.

Passive immunization with anti-CMV immunoglobulins has been the subject of several studies. The first studies were performed in bone marrow transplant recipients. Four studies on the effectiveness of CMV immunoglobulins were published [172 - 175]. In three of these studies a protective effect against CMV infection was reported [173 - 175]. However, the data of the Seattle bone marrow group [172] did not show a significant overall reduction of CMV infection, although in a small subpopulation (CMV seronegative recipients who did not receive granulocyte transfusions) a protective effect of the immunoglobulin preparation was observed.

In renal transplant recipients 4 studies on the use of CMV immunoglobulin preparations have been published [176 - 179]. Two studies were uncontrolled with variable results and the third was a prospective randomized trial in 59 CMV seronegative recipients of a CMV seropositive allograft [180]. In this study it was demonstrated that prophylactic use of CMV immune globulin provides substantial protection for primary CMV disease (reduction of CMV-associated syndromes from 60 percent in controls to 21 percent in recipients of CMV immune globulin). However no effect of immune globulin on rates of viral isolation or seroconversion was observed. Moreover, prophylactic effect of the globulin preparation was only seen in a subgroup of patients treated for rejection. The fourth study reports too the beneficial effect of anti-CMV immunoglobulin on decreasing the severity of primary CMV illness after renal transplantation. However, this study can be criticized because it lacked a concurrent control group [179].

In CMV seronegative heart and liver transplant recipients of a CMV seropositive allograft donor prophylactic given anti-CMV immunoglobulin did not influence the rate of CMV infection or disease (180, 181]. Both the studies were uncontrolled.

In conclusion, the results of the above mentioned studies are at variance and can be related to many variables such as patient selection, type and severity of immunosuppressive therapy, types of bloodtransfusion, sensitivity and specificity of viral surveillance procedures, and differences of antiviral activity, dosages and doses intervals of the anti CMV immunoglobulin preparations used. Moreover, most of these studies were uncontrolled, controlled with placebo preparation containing anti-CMV immunoglobulins, or open labelled studies.

Apart from antiviral chemotherapy adjustment of immunosuppressive therapy reduces remarkably the mortality and morbidity in case of CMV disease without an increased risk of graft failure or diminished graft function [182, 183].

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CHAPTER 2

AIM OF THE STUDY

Although the introduction of cyclosporin A (CsA) as the main immunosuppressive agent seems to have influenced the incidence and severity of CMV disease in a positive way, the reported incidence of clinical overt CMV infection is still 2 - 23 % and 1 - 3 % of the transplant recipients die from CMV infection. It is therefore obvious that this virus remains a major pathogen after organ transplantation.

DIAGNOSIS OF CMV INFECTION

When CMV disease is diagnosed, reduction of immunosuppressive therapy will markedly decrease morbidity and mortality without affecting graft survival. Moreover, antiviral therapy with either ganciclovir or foscarnet can be considered in patients with severe symptomatic disease. This management of symptomatic CMV infections (tapering of immunosuppressive drugs and / or antiviral therapy) makes rapid and early diagnosis necessary. Although the measurement of virus specific antibodies is sensitive, the long physiological response-time of antibody synthesis (one to two weeks) during active CMV infection makes this method inappropriate for rapid and early diagnosis. Moreover, in patients with immunosuppression antibody synthesis can be impaired. Detection of a morphological cytopathological effect (CPE) of CMV in tissue cultures has the same disadvantage. The method takes long time and is sometimes impossible due to bacterial contaminated specimens or coinfection with the herpes simplex virus.

In this thesis two methods for rapid and early diagnosis of CMV infection are described. First, we compared in our patients the results obtained by a low-speed centrifugation assay in combination with immunofluorescence by a monoclonal antibody against early antigen of CMV with the results from the conventional tissue culture method. Second, an indirect method for detection of active CMV infection is described. In the peripheral blood of renal transplant recipients mononuclear subpopulations were monitored with monoclonal antibodies before, during and after CMV infection.

HIGH RISK GROUPS

In chapter 4 we analyzed which transplant patients run a high risk for acquiring symptomatic CMV infection. First, an epidemiological study on the transmission of virus through kidney allograft and blood and its effect on graft and patient survival is described. Second, the impact of the use of rabbit-antithymocyte globulin in 49 renal transplant recipients and the incidence of CMV disease is studied.

PREVENTION OF CMV DISEASE

Ganciclovir has shown its efficacy in organ transplant recipients with severe CMV infections. However, the use of the drug is associated with severe side effects, e.g. neutropenia, especially in patients with impaired renal function. Moreover, the widespread use of ganciclovir may result in ganciclovir resistant CMV strains. Therefore, prevention of CMV infection is still preferred in organ transplant recipients especially in subgroups who are at high risk for CMV infection. There are three ways to prevent CMV infections in transplant recipients, as described in chapter 1. The first one is not to transplant organs or to transfuse bloodproducts from CMV seropositive donors into seronegative patients. The second possibility is prophylaxis with antiviral agents such as interferons, acyclovir or ganciclovir. The third option is to induce anti-CMV immunity, either actively or passively. In this thesis two studies on passive immunization in the two high risk groups are described.

First, the pharmacokinetics, safety and efficacy of an anti CMV immunoglobulin preparation were studied in CMV seronegative heart transplant recipients. The incidence of CMV disease in the untreated CMV seropositive and seronegative patients and the expected incidence based on the data from the literature were used as the reference group. In a subgroup of hearttransplant recipients CMV neutralizing antibody titers reached after infusion of the globulin preparation were determined. The second study is a double blind placebo controlled trial. Forty kidney transplant recipients, at risk because of rabbit antithymocyte globulin (RATG) treatment were randomly allocated to receive either immunoglobulin or a 20 % albumin solution. Both CMV seronegative and seropositive patients entered in this study, irrespective of the serological status of the allograft donor.

CHAPTER 3

MONITORING OF CMV INFECTION

3-1 RAPID DEMONSTRATION OF CYTOMEGALOVIRUS IN CLINICAL SPECIMENS

INTRODUCTION

Cytomegalovirus (CMV) infection is a major cause of morbidity and mortality in patients with impaired cellular immunity. Recipients of organ transplants [1,2] and patients with the acquired immunodeficiency syndrome (AIDS) [3] are at a particularly high risk of developing disseminated CMV infection. Rapid diagnosis of CMV infection is important in the management of these patients, because immunosuppression should be tapered or withdrawn in case of CMV disease [4, 5]. Moreover 9 (1,3-dihydroxy - 2-propoxymethyl) guanine (DHPG, Ganciclovir) has now become available for the treatment of CMV-infection [6 - 8].

The diagnosis of CMV infection classically depends on the isolation of virus or on serology. Many techniques have been applied for the detection of CMV antibodies, as e.g. seroneutralization, complement fixation, indirect hemagglutination and enzyme linked immunosorbent assay [9]. These techniques differ in specificity and sensitivity and measure antibodies directed against various CMV antigens. Unfortunately, the mere presence of antibodies against CMV antigens does not differentiate between present or past infection, although it has been suggested that antibodies to CMV-EA [10, 11] are a marker of an active CMV replication, which can take place in primary as well as in reactivated CMV infections. Furthermore it is suggested that the presence of IgM and IgE CMV antibodies could indicate recent primary infection [12, 13]. However, a rise of IgM CMV antibodies is observed in secondary infections too. Therefore the serological diagnosis of an active CMV infection still depends on a serological antibody titer rise, which may be observed 10 - 19 days after the onset of symptoms and thus is not useful for rapid diagnosis.

Virus isolation or more specifically, the detection of a characteristic cytopathic effect induced by the virus, when inoculated onto human fibroblasts is also a time-consuming method [14]. The cytopathic effect can be detected after 7 - 21 days of culture, but sometimes develops as late as 7 - 12 weeks after inoculation [15]. Considerable advances were made by using a centrifugation assay, adapted from the methods for chlamydial isolation [16, 17]. Specimens for CMV culture are spun down by low speed centrifugation on monolayers of susceptible cells. After 24 to 48 hours, the culture is ended and an immunofluorescence assay is performed in order to detect CMV early antigen (EA), using a monoclonal antibody.

In this study we compared the results obtained with the centrifugation assay with the results from the conventional tissue culture system.

MATERIALS AND METHODS

Cell culture systems.

Human embryonic lung (HEL) cells, passage 8-15 , were seeded (50.000 cells/ml, 1 ml per tube) in culture tubes (Flow Laboratories, Irvine, UK) or in disposable tubes with flat bottoms (Sterilin, Teddington, UK) and stored at 36° C in a maintenance medium of Dulbecco's modification of Eagles minimum essential medium (DMEM) containing 10 % foetal calf serum (FCS), 100 IU/L penicillin, 0.04 mg/ml streptomycin, 2.5 μg/ml amphotericin B, and 2 mM L- glutamine.

Processing of clinical specimens.

The clinical specimens used in this study were routinely submitted, in most cases for the follow-up of transplant recipients. They included urine, throat swabs, broncheoalveolar lavages (BAL) and blood buffy coats. After collection, throat swabs were immediately placed in maintenance medium (DMEM). Urine was centrifuged at 400 x g for 10 min. and brought on pH 7.2 with 0.09 M bicarbonate. Buffy coat cells were prepared from heparinized blood. Blood was centrifuged for 10 min at 900 x g and white cells were harvested. After removing erythrocytes as described by Roos and Loos [18], the cells were washed once in maintenance medium and suspended in 1.5 ml of the same medium. BAL were inoculated without further preparation.

Inoculation of clinical specimens and demonstration of CMV.

a) Centrifugation assay: After removal of the maintenance medium, 0.2 ml of the prepared specimen was inoculated directly onto the cell monolayer in two flat bottom tubes. The tubes were centrifuged at 900 x g for 1 hour at room temperature. After centrifugation 1.0 ml of the maintenance medium was added to each culture. The cultures were incubated at 36° C for 24 or 48 hours.

b) Detection: After incubation, the cover slip was removed from the tube and fixed in acetone for 10 minutes at room temperature. Next, the cover slips were rinsed with phosphate buffered saline (PBS) and incubated with a murine monoclonal antibody to CMV early antigen (M.A. Bioproducts Walkersville, Md, USA) in a dilution of 1:10 for 30 minutes at 36° C in a moist atmosphere. This procedure was followed by three washings with PBS and incubation with fluorescein isothiocyanate - labeled goat $(Fab)_2$- anti-mouse IgG (Tago, Burlingame,Ca., USA) in a dilution of 1:20. After three washings with PBS and one with distilled water, the cover slips were sealed in a glycerol buffer (Cityfluor, City University, London, UK). The cover slips were studied under a fluorescence microscope (Zeiss Epiillumination, Zeiss, Oberkochen, FRG). A specimen was considered positive if at least one intact cell with specific nuclear fluorescence (fig. 3-1) was detected.

c) Isolation of CMV in conventional cell culture: 0.2 ml of each clinical specimen was incubated for 1 hour on a HEL cell culture at 36° C. After discarding the inoculum, 1 ml medium (DMEM with 2 % FCS) was added and the cultures were incubated in a roller drum at 36°C. Cultures were examined twice weekly for CPE. The

whole culture period was six weeks. After three weeks, cells were tryptinized and inoculated in fresh culture tubes. After observing the characteristic CPE, CMV isolation was confirmed by an indirect immunofluorescence assay using pooled human serum containing high levels of anti - CMV antibody and goat anti - human IgG - FITC (Nordic, Tilburg, the Netherlands). Only cells with fluorescent nuclear inclusions were scored as positive.

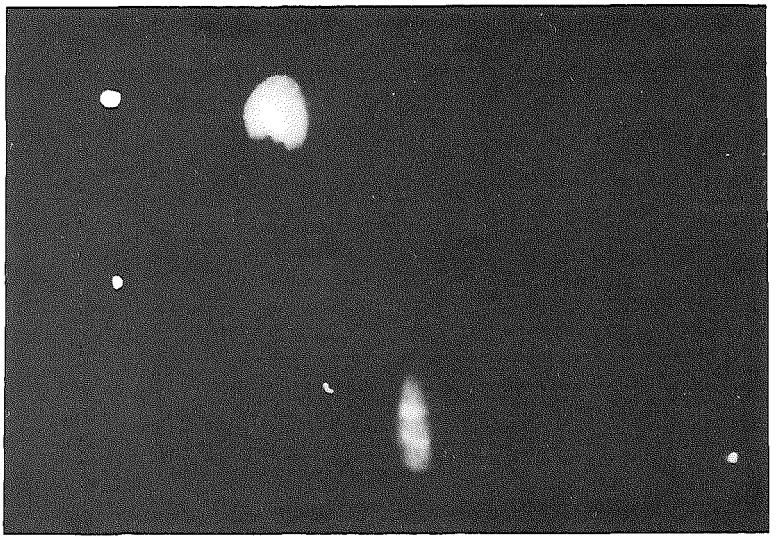

Fig. 3-1 Detection of CMV early antigen in a clinical specimen 24 hours after inoculation. Magnification 250 x.

RESULTS

A total of 155 specimens were obtained from 52 patients (table 3-1). In 14 out of the 52 patients (28 %) one or more specimens were found positive by at least one detection method (table 3-2). CMV was isolated in conventional cell culture from 31 specimens, all confirmed by immunofluorescence. The median duration of CPE to develop was 16,5 days (range 4 - 42 days). Twenty-eight of these 31 specimens were also found to be positive by the centrifugation assay. Three specimens were only positive for CMV in the conventional cell culture. These specimens were all buffy coats. On three occasions the centrifugation assay was found to be positive, whereas the cell cultures remained negative. However, CMV was cultured from other specimens obtained from the same patients before or at the same time. The remaining 121 specimens were negative both by the centrifugation assay and the conventional cell culture (table 3-3). The outcome of conventional virus culture in these specimens was: Herpes Simplex six times and adenovirus once (CPE and immunofluorescence); however, in the centrifugation assay, there was no reaction with the CMV monoclonal antiserum.

Materials	Number	Positive centrifugation assay (%)	Positive CPE (%)
Urine	68	24	22
Throatwash	58	19	17
Buffy-coat	24	8	21
BAL	5	20	20
Total	155	19	20

Table 3-1 **Materials from 52 patients studied for CMV isolation**
BAL = Broncheoalveolar lavage
CPE = cytopathogenic effect

DISCUSSION

The results of this study show that the centrifugation assay for detection of CMV-EA by immunofluorescence technique is a rapid, sensitive and specific method. Considering the conventional cell culture of CMV as a golden standard the centrifugation assay has a sensitivity of 91 % and a specificity of 97 %. The positive predictive value of the test is 90 % and the negative predictive value 97 %. The centrifugation assay was positive 14 days earlier than the conventional cell culture. A negative cell culture (CPE) with a positive result in the centrifugation assay does not exclude CMV infection. In all three cases, CMV was shedded from the patient in other specimens. This suggests that the centrifugation assay could be more sensitive than the conventional cell culture. On the other hand, the false negative results of the centrifugation assay were all from buffy coat cells. Cells which contain CMV in the blood are granulocytes, monocytes, and lymphocytes [19]. However, the virus is not present as a whole particle, but needs a final reassembly to achieve an infective capacity [15]. As a consequence viable cells are essential for isolation of CMV. It could be possible that centrifugation of white cells on the cell monolayer damages the cells and interferes with the infection capacity of CMV. Another explanation could be a low virus titer in the blood specimens, as is suggested by the fact that the CPE effect was observed after 4 - 6 weeks of inoculation of buffy coat cells of the centrifugation assay negative specimens in contrast to the 3 weeks in centrifugation assay positive blood specimens. This is in accordance with the observation of others [20 - 23] and suggests that virus titer could explain a negative centrifugation assay.

Disregarding the results of the blood specimens the sensitivity of the centrifugation assay approaches 100 % with a nearly 100 % negative predictive value. This is very important from both a clinical and a laboratory point of view, as a diagnosis of CMV infection can be made within 2 days.

Patient	Diagnosis	Specimen	Centrifugation assay 24 h	48 h	Cell culture +/-	Days
1	RT	Urine	+	+	+	25
		Urine	-	+	-	-
		Throat swab	-	+	-	-
		Urine	+	-	+	11
		Throat swab	+	-	+	28
		Buffy coat	+	+	+	26
2	RT	Urine	+	+	+	10
		Throat swab	+	+	+	10
3	HT	Urine	+	+	+	9
		Throat swab	+	+	+	9
		Buffy coat	-	-	+	26
		Urine	+	+	+	13
		Urine	+	+	+	10
4	RT	Urine	+	+	+	7
		Throat swab	+	+	+	11
		Urine	+	+	+	4
		Throat swab	+	+	+	7
5	Congenital CMV infection	Urine	+	+	+	16
6	RT	Urine	+	+	+	16
		Throat swab	-	+	+	16
		Urine	+	+	+	13
7	HT	Urine	+	+	+	27
		Throat swab	-	+	-	-
8	HT	Urine	+	+	+	22
		Throat swab	+	+	+	19
		Buffy coat	+	+	+	19
9	Prednisone therapy	Urine	+	+	+	8
		Urine	+	+	+	7
		Sputum	+	+	+	7
10	HT	Throat swab	+	-	+	23
11	RT	Throat swab	-	+	+	28
12	RT	Buffy coat	-	-	+	32
13	AIDS related complex	Buffy coat	-	-	+	44
14	AIDS	Bronchial washing	+	+	+	9
					mean	16.5

HT = heart transplant patient
RT = renal transplant patient

Table 3-2 Outcome of the centrifugation assay versus conventional cell culture in 14 patients with CMV infection.

		Cell culture	
		Pos	Neg
	Pos	28	3
Centrifugation assay			
	Neg	3	121

Table 3-3 **Comparison between CMV detection by the centrifugation assay and the conventional cell culture.**

The high specificity (97 %) of the centrifugation assay is in accordance with other studies [17, 23 - 27]. The centrifugation step is essential for a high sensitivity of the method, as is suggested by the reported sensitivity of 70 % in assays with monoclonal antibodies without centrifugation [27, 28]. The mechanism of the centrifugation assay to enhance the in vitro infectivity of CMV has still to be elucidated. This phenomenon was first described by Osborne et al [29] and was confirmed by Hudson et al [30, 31]. One hypothesis for the enhancement of infectivity of CMV by centrifugal inoculation is that the relatively low gravity promotes the contact between the virus and the target cells. Another hypothesis is that by centrifugation the "internal organization" of the cell, i.e. the structure of the membranes and organelles, is altered, causing a higher susceptibility to certain viruses.

Although the centrifugation assay has a high sensitivity, analysis of the blood specimens are still troublesome, possibly due to the low virus titer. Using density gradient method of leukocytes separation could result in a higher rate of CMV isolation.

In conclusion, the centrifugation assay makes a diagnosis possible the day after receipt of a specimen. This will help clinicians to instigate proper patient management early in CMV infection and reduce morbidity and mortality in these patients.

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3-2 MONONUCLEAR SUBSETS DURING CYTOMEGALOVIRUS DISEASE IN RENAL TRANSPLANT RECIPIENTS TREATED WITH CYCLOSPORIN AND RABBIT ANTITHYMOCYTE GLOBULIN

INTRODUCTION

The incidence of Cytomegalovirus (CMV) infection is high in immunosuppressed organ transplant recipients, especially in CMV seronegative recipients of a CMV seropositive allograft and in patients treated for rejection (see chapter 4). Although in many patients this infection runs an asymptomatic course, it may also give rise to clinical overt disease [1, 2]. The diagnosis of CMV infection through detection of virus-specific antibodies or virus isolation in cell culture is cumbersome and time-consuming and rapid viral diagnosis is obligatory in the management of transplant recipients. Apart from direct viral diagnostic methods as e.g. centrifugation assay with immunofluorescence of CMV-EA (see chapter 3-1) more indirect methods can be useful to detect imminent CMV infection. The use of monoclonal antibodies to determine mononuclear cell subpopulations may be helpful in the diagnosis of herpes infections after organ transplantation, especially in recipients under conventional immunosuppressive regimens, who do show true inversion of T helper/T suppressor-cytotoxic cells (CD4/CD8 <1.0) during virus infection [3, 4]. However, in patients on CsA and prednisone this phenomenon is thought to be less evident [5, 6]. This is a report of the effect of CMV infection on mononuclear subsets in a group of renal transplant recipients treated with CsA and low doses of prednisone.

PATIENTS AND METHODS

We studied clinical overt CMV disease and peripheral subpopulations of mononuclear cells in 49 consecutive renal transplant recipients, who were at risk for at least six months. The immunosuppressive regimen consisted of CsA, aiming at serum trough levels (conventional polyclonal RIA) of 100 - 200 mg/ml, and 15 mg of prednisone from the day of transplantation. Cyclosporin was introduced intravenous 6 hours after reanastomosis. In case of a biopsy proved allograft rejection rabbit antithymocyte globulin (RATG, Rijks Instituut voor de Volksgezondheid, Bilthoven, the Netherlands) was given [7]. In 21 patients a biopsy proved acute rejection episode was treated with rabbit antithymocyte globulin (RATG). Circulating T-cells (Leu-4 were kept between 50 - 150/mm^3 for 14 days. Clinical overt CMV disease was defined as fever of unknown origin for more than 2 days with another symptom and confirmed by isolation of CMV and/or a four-fold rise in antibody titre. Virus isolation and IgG antibody serology were performed as follows.

Virus Isolation

Urine samples and throat washings were inoculated onto human embryonic lung fibroblasts (Flow 2002, Flow Laboratories, Irvine, U.K.). Cultures were maintained for 6 weeks. All cultures with or without cytopathic changes were tested for CMV by an indirect immunofluorescence assay using pooled human serum containing high levels of anti-CMV antibody and goat anti-human IgG- FITC (Nordic, Tilburg, the Netherlands). Only cells with fluorescent nuclear inclusions were scored as positive [8].

Serology

Sera were separated and stored at -20° C until tested. The IgG class antibodies were determined by a twostep ELISA. CMV antigen was extracted from human embryonic lung cells (HEL) infected with CMV strain AD 169. The final antigen solution contained 9.5 mg protein per ml. Polystyrene 96-well microtitre plates (Greiner, Nurtingen, BRD) were incubated with 0.2 ml of the antigen diluted 1/800 with a 0.05 M bicarbonate buffer, pH 9.6. After overnight incubation at 4° C the plates were washed four times with phosphate buffered saline (PBS) 0.05 % Tween 20, pH 7.4.

In the ELISA duplicate solutions of sera were tested in five-fold dilutions from 1 to 100. PBS-Tween with 10 % fetal calf serum was used as dilution buffer (Flow Laboratories, Irvine, U.K.). The plates were incubated at 37° C during 2 hours, followed by three washes with PBS-Tween. For the second step a 30 minutes incubation was performed with 0.1 ml horse-radish peroxidase labeled goat anti IgG Fab-conjugate (Tago, Burlingham, USA), diluted with PBS-Tween 1:4.000. After three washes with PBS-Tween enzyme activity was demonstrated by a coupling-reaction with orthophenyldiamine (Abbott, Chicago U.S.A.) 1 mg/ml at room temperature, protected from the light. After stopping the reaction with 0.2 ml 4 N sulphuric acid, the extinction was read by a Titertek MCC/340 reader (Flow Laboratories, Irvine, U.K.) at a wave light of 492 nm. An extinction exceeding the negative controls plus twice the standard deviation was considered positive. The CMV IgM antibody test was performed by an indirect immunofluorescent method [9].

Mononuclear subsets monitoring

Blood samples were taken at regular intervals after grafting and more frequently during viral episodes. Peripheral blood lymphocytes (PBL) obtained after sedimentation of heparinized blood of Ficoll-Hypaque were stored at 70° C until tested. PBL were incubated with monoclonal antibodies of the Leu - series (Becton Dickinson (Leu-4 ;panT, CD3), Leu-3a (T helper, CD4), Leu-2a (T suppressor-cytotoxic, CD8), Leu-11 (Natural Killer cell, CD16), Leu-12 (B cells, CD19). Determinations were made by flow cytometry (Facs II, Becton Dickinson).

RESULTS

Rejection episodes were diagnosed in 23/49 (47 %) patients. In eight out of 49 patients (16 %), a clinical overt CMV disease was diagnosed ; all eight patients had received RATG treatment (see chapter 4 for more details). There were no patients with clinical signs of a CMV syndrome, without virological evidence for this infection. Clinical overt CMV disease was not found in any of the 28 patients without RATG anti-rejection therapy.

Period	Leu-4	Leu-3a	Leu-2a	Leu-3a/Leu-2a
Before (1-4 weeks)	57 (32-66)	40.5 (35-47)	17 (12-27)	2.7 (1.6-4.1)
Before (0-1 weeks)	63 (52-68)	22.5 (20-37)[a]	38 (30-45)[b]	0.7 (0.4-1.0)[a]
During	61 (36-68)	21 (15-29)[a]	48.5 (30-59)[a]	0.5 (0.3-1.0)[a]
After	55 (54-64)	45 (26-48)	21 (16-29)	1.7 (1.2-2.7)

Table 3-4 **Percentages mononuclear subsets (Leu-serie) (Median-Range) 1-4, 0-1 weeks before, during and after Cytomegalovirus Disease.**
*** All subsets are compared with the percentages of the 1-4 weeks period**
[a]Wilcoxon-test, $\alpha < 0.01$
[b]Wilcoxon-test, $\alpha < 0.05$

	Lymphocytes	Leu-4	Leu-3a	Leu-2a
Before CMV	996 ± 542	527 ± 370	418 ± 306	205 ± 92
		$p < 0.01$ *		$p < 0.01$
During CMV	1633 ± 879	1048 ± 552	248 ± 134	934 ± 536

Table 3-5 **Absolute number of circulating lymphocytes per cubic millimeter (means ± SEM) of the Leu-4, Leu-3a, and Leu-2a phenotypes before and during CMV disease.**
*** $p < 0.01$, unpaired student-t test.**

Table 3-4 shows the results of mononuclear subsets monitoring in six patients with symptomatic CMV infection. In all of these patients a true inversion of CD4/CD8 ratio was already apparent one week before the onset of symptoms. Lymphocyte - subset counts before and during symptomatic CMV infection are presented in table 3-5. During CMV infection there is a T cell lymphocytosis and the decreased CD4/ CD8 ratio is due to a large - and significant increase in the suppressor-cytotoxic T

cell phenotype, with a slight reduction in helper T phenotype. There was no correlation between CMV disease and the percentage of cells positive for CD16 or CD19 in this group of patients.

Two patients, treated for rejection with RATG developed a reduction of CD4/CD8 ratio (< 1.0) in the absence of CMV disease; both had other symptomatic viral infections. In the 26 patients who were not treated for rejection, we observed only three other individuals with inverted ratios. One had both a severe symptomatic herpes homines infection and only serological evidence for CMV reactivation. In the other two patients no viral infection was diagnosed.

DISCUSSION

It has been reported that in transplant recipients under CsA therapy true inversions of CD4/CD8 ratio occur infrequently during CMV infection [5, 6, 10]. Nevertheless, in one of the reports three of the four symptomatic patients in fact did show this phenomenon [6]. So it seems that in clinical overt CMV infections a more pronounced alteration in T cell subsets occurs. In the present study CsA-treated patients with clinical overt CMV disease had inverted ratios, suggesting that T cell subsets during CMV illness show the same pattern in normal adults [11] and in transplant recipients, irrespective of the immunosuppressive agents used. As the fall in CD4/CD8 ratio is already apparent one week before the first symptom developed the ratio appears to predict a forthcoming CMV infection. However, a decrease of the CD4/CD8 ratio is also noticed in other herpes virus infection [3], as well as during nonherpetic viral infections [12,13]. In conclusion, monitoring mononuclear subsets in the peripheral blood can be of help in diagnosing viral infections after organ transplantation.

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CHAPTER 4

IDENTIFICATION OF ALLOGRAFT RECIPIENTS AT HIGH RISK FOR CMV DISEASE.

4-1 RABBIT ANTITHYMOCYTE GLOBULIN INCREASES THE INCIDENCE OF CMV RELATED MORBIDITY.

INTRODUCTION

Cytomegalovirus (CMV) infection is a well known complication in renal transplant recipients treated with azathioprine and steroids. In many patients this infection gives rise to clinical illness with fever, leucocytopenia, thrombocytopenia, hepatitis, interstitial pneumonitis and gastrointestinal bleeding. Renal allograft glomerulopathy, superinfections with other opportunistic agents and possibly Kaposi's sarcoma are other consequences of CMV infection [1,2]. The use of immunosuppressants promotes the development of this infection, as an intact cellular defense mechanism is essential for anti-CMV immunity. It has been claimed that under cyclosporin (CsA) therapy the incidence of CMV disease is lower than under conventional immunosuppressive treatment [3 - 6], but others were not able to confirm this [7 - 9]. The discrepancy may be related to differences in concomitant immunosuppressive schedules e.g., the dose of steroids or antithymocyte globulin (ATG). We studied the incidence of clinical overt CMV infection in renal allograft recipients under cyclosporin and low dose prednisone.

PATIENTS AND METHODS

We studied clinical symptomatic CMV disease in 49 consecutive renal transplant recipients, who were at risk for at least 6 months. The immunosuppressive regimen consisted of CsA, aiming at serum trough levels (conventional polyclonal RIA) of 100 - 200 mg/ml, and 15 mg of prednisone from the day of transplantation. Cyclosporin was introduced i.v. 6 hours after reanastomosis. In case of a biopsy proved allograft rejection rabbit antithymocyte globulin (RATG, Rijks Instituut voor de Volksgezondheid, Bilthoven, the Netherlands) was given [10]. Circulating T-lymphocytes (Leu-4, Becton-Dickinson) were kept between 75 - 150/mm^3 for 14 days. Clinical overt CMV disease was defined as fever of unknown origin for more than 2 days with another symptom like leucocytopenia, thrombocytopenia, hepatitis or interstitial pneumonitis and confirmed by isolation of CMV and /or a four-fold rise in CMV antibody titer. Virus isolation, IgG and IgM antibody serology were performed as described in chapter 3.

Statistical methods

For statistical analysis the Wilcoxon rank test and the Chi-square test were used.

Patient number	Fever	Leuco-penia	Thrombo-penia	Hepatitis	Anti-CMV IgG before	Anti-CMV IgG after	IgM	Viral culture
1	+	+	+	-	< 100	2.429	ND	-
2	+	+	-	-	< 100	796	+	+
3	+	+	-	+	3.874	10.675	+	+
4	+	+	+	+	1.462	7.522	-	-
5	+	+	+	-	714	13.562	-	-
6	+	-	+	-	2.481	13.246	+	+
7	+	-	+	-	2.625	8.127	-	-
8	+	+	-	+	< 100	796	+	+

Table 4-1 Clinical and virological features of 8 patients with CMV disease.

RESULTS

Rejection episodes were diagnosed in 23/49 patients (47 %). In eight out of 49 patients, a clinical overt CMV disease was diagnosed; all eight patients had received RATG treatment. Clinical overt CMV disease was not found in any of the 26 patients on CsA and low dose prednisone without RATG anti-rejection therapy (table 4-1). All 8 patients had fever and leucocytopenia and/or thrombocytopenia and three patients had hepatitis. In all patients a more than four-fold rise in titre of anti-CMV IgG (ELISA) was found and in four patients CMV could be isolated from urine or throatwash. Symptoms developed 3 weeks (median, range 3-5) after the first RATG infusion and fever lasted for 5 (median, range 2-15) days. No CMV-related deaths were recorded. There were no patients with clinical signs of a CMV syndrome as defined above, without virological evidence for this infection. Patients with CMV disease did not have more days in which circulating T cells were below 150/mm^3 as a consequence of RATG treatment than patients who did not show CMV disease after RATG. However, the dose/kg bodyweight RATG given to patients who developed CMV infection was significantly higher (Wilcoxon rank test $p < 0.05$) compared with the dose in patients without clinical infection (14.9 mg/kg, range 9.6 - 24.9 versus 11.7 mg/kg, range 4.0 - 29.0). Figure 4-1 shows the correlation between the overall incidence of CMV disease and percentage of renal transplant patients treated with anti-lymphocyten globulines (ALG) in seven studies including the above described data.

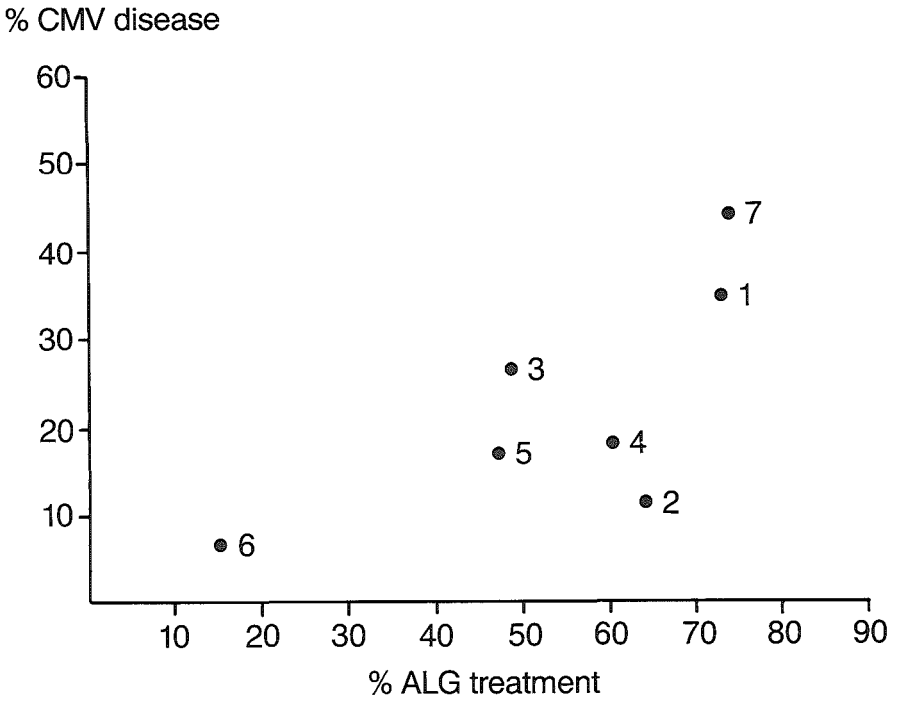

Fig. 4-1 **Correlation between the overall incidence of CMV disease and percentage of renal transplant patients treated with ALG in 7 studies. Each dot represents one study. 1. Pass et al. (11) 2. Bia et al. (12), 3. Rubin et al. (13) 4. Hoitsma et al. (14), 5. Present study 6. Weir et al. (15), 7. Snydman et al. (16).**

DISCUSSION

This study indicates that renal transplant recipients treated with CsA and low dose prednisone alone are not at great risk of attracting CMV disease. However, when RATG was added to this protocol because of rejection crises, CMV-related morbidity was found in 8/21 (38 %) patients. Moreover, the incidence of CMV infection was related with a higher cumulative dose of RATG/kg bodyweight , which is in agreement with the observation that after gifts of lower doses ATG the incidence of CMV infection is diminished in renal transplant recipients on azathioprine [11]. When ATG treatment was given as rejection prophylaxis in addition to azathioprine an increased incidence of CMV infection was observed too [17]. However the observation of Rubin et al [13] that CMV infection rate did not increase in ATG treated patients when the concomitant conventional therapy had been halved, suggests that the net state of immunosuppression is more important than the specific agents used. This is in agreement with the report of Hoitsma et al [14], in which no difference was seen in incidence of CMV disease in renal allograft recipients treated for rejection with

steroids or with the same RATG preparation used by us. In the latter group, on azathioprine and prednisone as standard immunosuppression, the incidence of CMV disease (32 %) was comparable with the results in the present study. Apparently, after anti-rejection therapy with RATG the incidence of CMV infection is independent of the use of cyclosporin or azathioprine. Another support for the net state of immunosuppression as the main cause for CMV disease comes from the observation of Velasco et al [18]. In a retrospective study in 92 cadaveric renal allograft recipients CMV infection was more frequently observed in patients treated with high dose of steroids as compared with those treated with low doses of steroids.

In conclusion, transplant recipients on CsA and low dose prednisone are not at great risk to develop clinical overt CMV infection. Additional immunosuppression with RATG is associated with considerable CMV related morbidity. The overall incidence of CMV disease depends on the percentage of patients treated with ALG in a given population.

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4-2 PREVENTION OF CMV INFECTION BY SCREENING FOR CMV ANTIBODIES IN RENAL ALLOGRAFT RECIPIENTS AND THEIR BLOOD AND KIDNEY DONORS.

INTRODUCTION

CMV infection remains a major cause for morbidity and mortality in renal transplant recipients both under conventional immunosuppression (AZA) and cyclosporine (CsA). CMV disease may result from reactivation of latent virus in the host or from transmission of the virus with the transplanted kidney [1,2] and blood products [3] from CMV infected donors. Since CMV infections in seronegative kidney transplant recipients run a serious clinical course associated with reduced graft - and patient survival [4] prevention of virus transmission is still warranted. As accurate and rapid assays for the screening of CMV antibodies are now available, the CMV serostatus could be taken into account during donor-recipient selection. However, the influence of CMV infection on graft survival remains a controversial issue [5]. Furthermore the serologic status of blooddonors was not monitored in most studies examining the relationship between CMV acquisition following renal transplantation and bloodtransfusion [6]. Therefore a prospective study was initiated in two Dutch transplantation centers to assess the relevance of the CMV serological status of blood - and kidney donors on graft and patient survival.

PATIENTS AND METHODS

We studied 73 patients (median age 44, range 18 - 64 years) who received a cadaveric donor kidney in 1983 and 1984 with the services of the Eurotransplant Organization, Leiden, the Netherlands. Ten patients received a second and two patients a third allograft. All recipients were treated with azathioprine and prednisone and all received blood transfusions prior to transplantation. Symptomatic CMV disease was defined as otherwise undefined fever (> 38.5° C) for more than two days with another symptom like leucocytopenia, thrombocytopenia, hepatitis or interstitial pneumonitis, and confirmed by isolation of CMV and/ or a significant rise in antibody titer. IgG antibodies against CMV were determined by an enzyme-linked immunosorbent assay (ELISA) as described in detail elsewhere [7]. Sera with >8.0 ELISA units were considered to be positive for CMV. Significant differences between paired sera, indicating recent infection, where defined by a more than 2.5 fold increase in ELISA units. Serum samples, urine and throat washes were obtained from each patient pre-transplantation and bi-monthly during the first 5 months after transplantation. Culture samples were inoculated onto human embryonic lung fibroblasts and cultures were maintained for 6 weeks and screened for cytopathic changes. Specific anti-CMV immunofluorescence was performed on all cultures. One serum sample of the kidney donor and plasma samples of all units blood, given in the first 5 months after transplantation were screened for CMV antibody by ELISA. In all cases leucocyte-free blood, filtered within 24 hours after donation through a cellulose-acetate filter was used. Graft loss was defined as the need to reinstitute chronic hemodialysis, transplant nephrectomy, or death with or without renal failure. Graft and

patient survival were calculated with the actuarial life table method. The statistical analysis was performed with the Chi-square test and the Fisher exact probability test.

RESULTS

CMV antibodies were detected in 54 of 73 (74 %) renal transplant recipients at the day of transplantation and in 49 of 73 (67 %) kidney donors. Donor - recipient combinations were consequently divided into four groups according to the presence or absence of anti-CMV antibodies (table 4-2). Thirty-seven of the 54 CMV seropositive recipients received a kidney from a CMV seropositive donor and 17 patients received a kidney from a CMV seronegative donor. In 19 renal allograft recipients no CMV antibodies were found at the time of transplantation, of whom 12 received a kidney from a CMV seropositive donor and 7 from a CMV seronegative donor. Almost all renal allograft patients (93 %) received one or more units of blood (median 2, range 1 - 17) during the first 5 months after transplantation. Table 4-2 shows the number of patients receiving blood from CMV seropositive donors.

CMV serostatus donor/recipient	No. of pat.	% recipients of CMV pos. blood
Positive/positive	37	65
Negative/positive	17	76
Positive/negative	12	58
Negative/negative	7	71

Table 4-2 **Number of donor/recipient combinations and the percentage of recipients with CMV seropositive blood transfusions**

Out of 7 CMV seronegative kidney donor - recipient pairs 5 patients received blood from CMV seropositive donors. Thirteen out of 17 CMV seronegative kidney donor/CMV seropositive recipients pairs received blood from CMV seropositive donors. Table 4-3 shows the incidence (%) of CMV seroconversion and CMV disease in the 4 donor-recipient subgroups. Primary CMV infection was observed in 8/19 (42 %) CMV seronegative patients. All 8 patients had received grafts from CMV seropositive donors. Consequently 8 out of 12 (67 %) CMV seronegative patients who obtained grafts from CMV seropositive donors developed CMV infection. CMV associated symptoms were observed in 6 patients. Three patients developed an interstitial pneumonia, of which two were fatal. In contrast, no primary CMV infection was observed in the group of CMV seronegative donor - recipient pairs, although 5 patients had received blood from CMV seropositive donors after transplantation. This difference between the two subgroups was statistically significant ($p < 0.01$). Secondary CMV infection was observed in 29 out of 54 (54 %) CMV seropositive patients.

Donor/recipient serostatus	Number	Infection Number(%)	Disease Number(%)	Death Number(%)
Positive/positive	37	19 (51)	7 (19)	0
Negative/positive	17	10 (59)	2 (12)	0
Positive/negative	12	8 (67)	6 (50)	2 (17)
Negative/negative	7	0	0	0
Overall	73	37 (51)	15 (21)	2 (3)

Table 4-3 **Incidence of CMV infection, disease and CMV related death in 4 donor/recipient combinations, according to the CMV serostatus CMV antibody status as defined by the ELISA test.**

In 9 of the 54 patients (17 %) symptomatic illness was seen, but no mortality was found in this group. The incidence of CMV infection in the CMV seropositive allograft recipients was not influenced by the CMV serostatus of kidney and blood donor. CMV infection symptoms was associated with clinical illness in a significantly higher percentage in primary infections than in secondary infections (75 vs 31 %, $p < 0.05$).

Graft survival data of the two CMV seronegative recipients subgroups are shown in figure 4-2. A higher graft survival rate (72 % at 3 years) was observed in the CMV seronegative donor-recipient group when compared to the 41 % graft survival rate at 3 years in the CMV seropositive donor / CMV seronegative recipient group. This poor graft survival rate was observed only in patients who suffered from CMV infection. The CMV seronegative recipients with CMV seropositive donors, who remained free from CMV infection, showed the same graft survival (75 % at three years) as the CMV seronegative donor / recipient subgroup. Four CMV seronegative recipients with CMV seropositive kidney donor lost their graft due to rejection while only one CMV seronegative recipient of a CMV seronegative kidney had an irreversible rejection. The patient survival rate in the CMV seronegative donor / recipient subgroup was 100 % 3 years after transplantation. As the result of CMV related mortality in 2 patients the actuarial 3 years patient survival was 83 % in the CMV seropositive donor / CMV seronegative recipient subgroup.

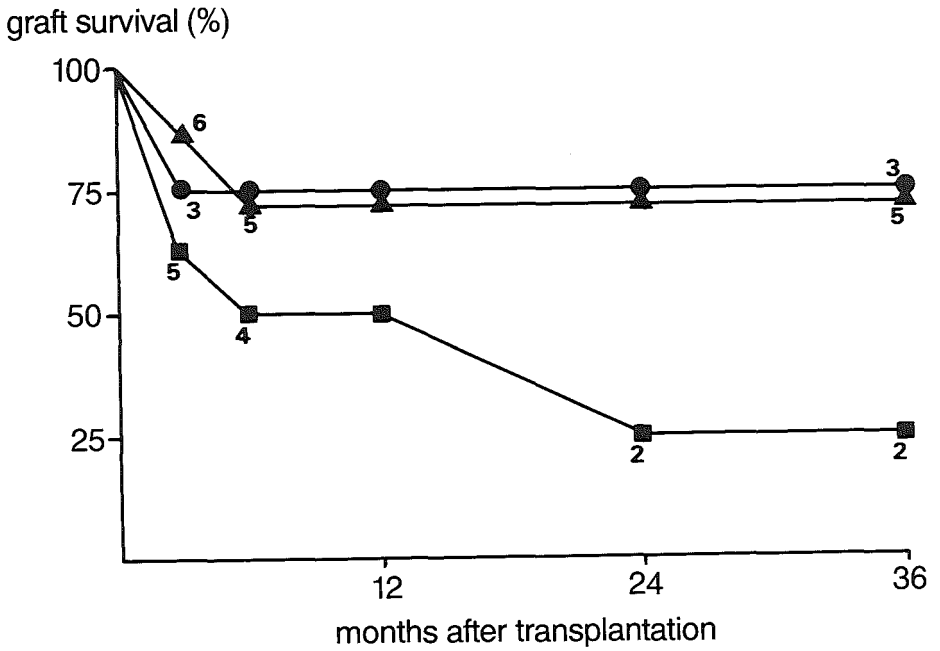

Fig. 4-2 **Actuarial kidney graft survival of 8 CMV seronegative recipients with CMV seropositive donors and CMV infection (▪—▪), 4 CMV seronegative recipients with CMV seropositive donors without CMV infection (●—●) and 7 CMV seronegative donor-recipient pairs (▲—▲)**

DISCUSSION

Blood transfusions are a possible source of CMV. It has been calculated that 2.5 to 12 % of all transfusions result in virus infection in the immunocompetent patients [3,8,9,10]. However immunocompromised patients can acquire CMV infection in a much higher incidence [11]. The risk of acquiring CMV infection varies with the donor population and with the type and storage of blood products [12]. In this study transmission of CMV via bloodproducts was not observed. Five out of 7 CMV seronegative recipients with CMV seronegative kidney donors received 1 - 2 units of leucocyte free blood from CMV seropositive donors. None of the 5 patients developed CMV infection. Furthermore, 4 of the 12 CMV seronegative recipients with CMV seropositive kidney donors remained free of CMV infection, despite the fact that these patients received blood from CMV seropositive donors. An explanation for the lack

of transmission of CMV with blood could be due to the removal of white cells, although the group at risk may be too small to make definite conclusions.

This study confirms epidemiological studies in the USA and UK concerning the potential risk of CMV transmission through transplantation of renal allograft obtained from CMV seropositive donors [1, 2, 13 - 16). This is not quite unexpected since CMV could be isolated from renal allograft tissue [17]. The incidence of CMV infection, 8 out of 12 (67 %) and CMV related morbidity, 6 out of 8 (75 %) was high in the CMV seronegative recipients with CMV seropositive kidney donors. Furthermore, mortality was confined to this subgroup only. In contrast, none of the CMV seronegative patients who received an allograft from a CMV seronegative donor developed a CMV infection. On the other hand CMV infection in CMV seropositive recipients was independent of the CMV serostatus of the kidney donor.

In the present study we also demonstrated that the group of CMV seronegative recipients with CMV seropositive kidney donors had a lower long term graft survival rate when compared to the CMV seronegative kidney donor/recipient pairs. Although this is in agreement with others (18,19), such a detrimental influence of the CMV serostatus of the organ donor on graft and patient survival of CMV seronegative recipients is certainly not an unanimous finding [5,13,20 - 22]. This discrepancy could be due to the prevalence of CMV seropositivity in the donor and recipient populations studied and the retrospective vs prospective nature of the various studies. Furthermore, the differences in the serological methods used could influence the distribution of the CMV serostatus dependent classification of donor/recipient pairs and the diagnosis of CMV infection. Other factors influencing the outcome of kidney transplantation, e.g. HLA matching, pretransplant blood transfusions and ischemia times could attribute to the differences of the influence of CMV infection on graft survival.

In conclusion, due to the high prevalence of CMV antibodies in our donor - and acceptor population (67 and 74 % respectively) only a minority (16 %) of the total group of recipients acquired CMV infection through virus transmission with the allograft. However, in these patients a high incidence of CMV related morbidity is found and graft survival is considerably decreased. As accurate and rapid assays for the screening of CMV antibodies are now available selection of CMV seronegative kidney donors for CMV seronegative recipients has become feasible and could improve graft and patient survival.

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CHAPTER 5

PROPHYLACTIC USE OF ANTI-CMV IMMUNOGLOBULIN IN CMV SERONEGATIVE HEART TRANSPLANT RECIPIENTS

INTRODUCTION

Cytomegalovirus infection remains a major problem after clinical heart transplantation. Especially primary infections may result in serious morbidity and even mortality. Primary infections result from transmission of the virus with an allograft or with bloodproducts from CMV seropositive donors into CMV seronegative recipients. The incidence of CMV disease in the CMV seropositive heart donor/seronegative recipient combination has been reported to be 64-92 %. In the seronegative heart recipients from a seronegative donor the incidence is still 15 % [1-3]. Avoidance of CMV transmission by selecting CMV seronegative allograft and blooddonors for CMV seronegative recipients will prevent primary CMV infection after transplantation. However this strategy is not always logistically feasible and it can prolong the time on the waiting list, which is often unacceptable for critically ill heart transplant candidates. Consequently, other methods to prevent CMV infection have to be evaluated. Prophylactic use of antiviral agents is a possibility. However, reports on the efficacy of acyclovir are controversial [4-6] and interferon α nor interferon ß reduced the incidence of CMV disease, while interferon α was associated with severe acute rejections [7-11]. Moreover, the widespread use of ganciclovir, although effective in the treatment of CMV disease, could result in ganciclovir resistant CMV strains [12] and in myelosuppression associated superinfections. Active immunization of seronegative kidney transplant candidates with an attenuated live CMV strain did not prevent CMV infection nor CMV disease after transplantation, although the symptomatic infections did run a milder course in these patients than in their seronegative non-immunized controls [13-14].

Passive immunization with anti-CMV immunoglobulin preparations reduced the severity of CMV disease in seronegative kidney recipients from seropositive donors [15-16]. These studies confirm earlier observations in bone marrow transplant recipients [17-19]. The value of passive immunization for heart transplant recipients is unclear as only limited and inconclusive results have been published on this subject [20]. However, even when efficacy in prevention of CMV infection can be demonstrated, concern is aroused about the potential transmission of other viruses, e.g. human immunodeficiency virus (HIV), Hepatitis non-A, non-B virus and Hepatitis B virus (HBV) through these blood products. Furthermore, the use of globulin products has been associated with hemodynamic side-effects. Therefore it was decided to accrue arguments for the efficacy and safety of passive immunization in CMV seronegative heart transplant patients, in which the expected incidence of disease is high, especially in case of a CMV seropositive donor (36/44, 82 %) [1-3].

PATIENTS AND METHODS

During a period of 4 years 78 heart transplantations were performed in 77 patients. The immunosuppressive regimen consisted of cyclosporin A (CsA) and prednisone. The dose of CsA was adjusted according to plasma trough levels. Endomyocardial biopsies were performed at regular intervals. In case of a biopsy proved rejection 3 times 1 gram of methylprednisolone was administered intravenously. In steroid unresponsive rejections rabbit anti-thymocyte globulin (RATG, National Institute for Public Health, Bilthoven, the Netherlands) was given. During this treatment peripheral T cells ($CD3^+$) were kept below $150/mm^3$ during 3 weeks.

The CMV serostatus of the transplant recipients was screened for anti-CMV IgG by an ELISA (see chapter 3 for details). Recipients with a pretransplant ELISA titer <100 were considered to be CMV seronegative. Serum of allograft donors was retrospectively screened for CMV IgG antibodies too. Blooddonors were not screened for CMV IgG antibody, but peri-, per- and post transplantation only buffy-coat depleted blood was given.

After the ninth transplantation all CMV seronegative recipients received anti-CMV immunoglobulins, irrespective of the CMV serostatus of the allograft donor. A commercially available immunoglobulin preparation was used (Cytotect, Biotest Pharma GmbH, Frankfurt, FRG). It was produced by cold ethanol precipitation of plasma pools with high titers of antibodies to CMV. Sterilization was performed by ß-propiolactone treatment [21]. The preparation contained 100 mg protein/ml and had a specific IgG antibody level of 40.000 ELISA units/ml (50 U/ml ELISA against the Paul Ehrlich standard). The CMV neutralizing antibody titer was 1:3000 [22]. The first gift of immunoglobulin was infused in a dose of 150 mg/kg body weight during transplantation before recirculation started. Thereafter, on day 2, 7, 14, 35, 56 and 77 the same preparation was given in a dose of 100 mg/kg. The immunoglobulin, diluted in 250 ml of saline, was infused at a rate of 1-2 ml/min.

Before each infusion of globulin, samples of urine, throatwash and blood were collected for virus isolation. When indicated more specimens were obtained for diagnosis. The isolation of CMV was performed by a low-speed centrifugation assay in combination with immunofluorescence using a monoclonal antibody against early antigen of CMV, as described in detail in chapter 3. Buffy-coat samples were also cultured on human embryonic lung fibroblasts and screened for cytopathic change. Specific anti-CMV immunofluorescence studies were done on all cultures. From the CMV seropositive recipients and from the untreated CMV seronegative recipients clinical specimens were obtained monthly or more frequently when infection was suspected.

Symptomatic CMV infection was defined as illness with two of the following symptoms without other possible explanations: fever(> 38.5°C) for at least 3 consecutive days, gastrointestinal, lung or central nervous system involvement, leucocytopenia (< 3.0×10^9/L), thrombocytopenia (< 100×10^9/L), elevated serum alanine or aspartate aminotransferases (> 2.5 times the upper limit of normal). This viral syndrome had to be confirmed by concomitant isolation of CMV. Organ involvement had to be confirmed by culture or biopsy from the diseased organ. In case of serious CMV disease therapy with ganciclovir (9-[2-hydroxy 1-(hydroxymethyl)ethoxymethyl]guanine, DHPG, Sarva Syntex, Maidenhaid, U.K.) was instituted.

The incidence of CMV disease in the untreated CMV seropositive and seronegative recipients and the expected incidence based on the data from the literature (Med Line search up to July 1989) were used as the reference group. For differences between the globulin and the reference group the point estimate and its 95 % confidence interval (CI) are given. To test the hypothesis that there was no difference between these groups the chi-square test was used. The results of tests of significance are reported as two tailed.

In a subgroup of eight CMV-seronegative recipients the pharmacokinetics of the globulin preparation were studied. Pharmacokinetics were performed by comparing calculated log ELISA titers over time. The T1/2 was determined by graphing the log of the concentration vs time in days. The T1/2 is related to the slope of the line by the equation T1/2 = ln/K, whereas K is the slope constant and equated with 2.3 (log conc 2-log conc 1)/ time interval [23].

In fifteen CMV seronegative recipients the total and neutralizing CMV IgG antibody levels induced by the infusion of the globulin preparation were studied in correlation with its efficacy in preventing CMV infection and disease. Before and after each infusion CMV IgG and CMV neutralizing antibodies were determined. CMV IgG was measured with an ELISA, as described in detail in chapter 3. CMV neutralizing antibodies were determined with a fluorescing cell assay [24-25]. This assay was performed in triplicate with serial two fold serum dilutions in maintenance medium (DMEM, Flow Lab) supplemented with 2.5 % quinea pig serum and mixed with an equal volume of virus (AD 169 CMV strain) suspension, yielding 200 fluorescent cell units (FCU)/0.1 ml in absence of neutralization. After incubation for 30 minutes at 37°C the mixture was replaced with fresh medium and incubated for 18 hours at 37° C. Thereafter fluorescent cell assay in quadruplicate with a monoclonal antibody against early antigen of CMV (EA, Dupont de Nemours, Inc., Wilmington, DE) was performed. The neutralizing antibody titer was defined as the reciprocal serum dilution giving 100 FCU. All titers are given as geometric mean titers (GMT).

Potential adverse effects during and after infusions were recorded. We studied the effect of the second globulin gift on hemodynamic parameters at the second postoperative day. Patients were continuously monitored with indwelling left atrium, central venous and arterial pressure lines. Moreover the heartrate was recorded. Filling pressures, blood pressures and heart rate were recorded at the start of globulin infusion and at 1, 2, 3, and 4 hours thereafter.

Before and after each globulin infusion and bimonthly after the last dose serum of the patient was screened for HBsAg (ELISA, Abbott Lab, USA), HIV antibodies (Rec HTLV 3 EIA, Abbott Lab, USA) and alanine aminotransferase and aspartate aminotransferase. Sera of all blood and organdonors were screened for IgG antibodies against HIV and HBsAg. In case of HIV seropositivity with the EIA Western immunoblot assay was performed. Transmission of virus was defined as detectable HBsAg, antibodies against HIV or otherwise unexplained elevation (> 2.5 times upper limit) of the liver enzymes.

RESULTS

1. Prevention of CMV infection and disease.

In Table 5-1 the characteristics of the 77 heart transplant recipients are shown. 41/77 (53 %) patients were CMV seropositive at the time of transplantation. Twenty of the 36 CMV seronegative patients received an allograft from a seropositive donor.

	CMV SEROPOSITIVE	CMV SERONEGATIVE
Sex male	**34**	**35**
female	**7**	**1**
Age, years	**46**	**41**
(median, range)	**(29 - 56)**	**(12 - 55)**
≥ 1 anti-rejection therapy	**24**	**22**
RATG-therapy	**7**	**9**
Death ≤ 14 days after Tx	**3**	**3**

Table 5-1 **Characteristics of 77 heart transplant recipients divided in two-groups according to their CMV serostatus.**

One CMV seronegative patient received two allografts, the first from a CMV seronegative and the second from a CMV seropositive donor. Six out of the 77 patients died within 14 days after transplantation because of a non-infectious complication and were excluded from further analysis. In 46 (65 %) of the remaining patients at least one anti-rejection therapy was instituted. Fifteen patients were treated with RATG. The follow-up in the 71 patients was 6 - 39 (median 19) months after transplantation.

Table 5-2 shows the incidence of CMV isolation and disease in 4 groups of heart transplant recipients, according to the CMV serostatus of donor/recipient and passive immunization. One out of 16 treated seronegative heart recipients from a seronegative donor developed symptomatic CMV infection at 10 weeks after transplantation, one week after the start of anti-rejection therapy with RATG. In 8/16 (50%) treated CMV seronegative heart recipients from a seropositive donor CMV could be isolated at a median of 40 (range 17 -240) days after transplantation. In seven patients CMV was isolated from the blood. Three of these patients had been treated with RATG. Two of them developed CMV related symptoms. In both patients lunginvolvement was present and one patient was treated with DHPG. None of the symptomatic patients died because of CMV infection.

Another of the globulin treated CMV seronegative heart recipients a CMV seropositive donor developed CMV related symptoms 27 weeks after transplantation, while the anti-CMV IgG serum level had decreased < 100. During the immunoprophylactic period this patient was viremic without accompanying symptoms. The incidence of CMV isolation in the 38 seropositive recipients was 42 % . The first isolation of CMV isolation was observed at a median of 47 (range 7- 300) days after transplantation. In 4 patients viremia was diagnosed and all 4 developed CMV related

	GLOBULIN TREATMENT		NO TREATMENT	
CMV SEROSTATUS	NEG/NEG	POS/NEG	/POS	POS/NEG
DONOR - RECIPIENT	(N=16)	(N=16)	(N=38)	(N=2)
CMV isolation	1 (6)	8 (50)	16 (42)	1 (50)
CMV disease	1 (6)	2 (13)	4 (11)	1 (50)
CMV lung involvement	0	2 (13)	1 (3)	0
CMV related death	0	0	1 (3)	0

Table 5-2 **Number (%) of patients with CMV isolation, disease, lung involvement and related death in 4 groups of heart transplant recipients, according to the CMV serostatus of donor/recipient and globulin treatment.**

disease. Two patients were treated with DHPG. One patient died because of CMV lung involvement. One of the two untreated CMV seronegative recipients from a CMV seropositive donor developed CMV disease.

Based on the data from the literature the expected incidence of CMV disease in CMV seronegative recipients from a CMV seropositive donor was 82 % (36/44). This would mean for our study group 13 patients with CMV disease. The observed incidence was 2/16 (13 %). The difference between observed and expected incidence was 69 % (95 % CI 42 - 97 %, $p < 0.001$)

2. Pharmacokinetics.

All 8 patients developed high peak CMV antibody titers after the first infusion of median 5.100 (range 1.100-8.400). The peaklevels after the second infusion had a range of 1.700 to 6.700 (median 3.000) ELISA units. After the third and fourth infusion the peak levels were comparable. As shown in fig. 5-1, the T1/2 of disappearance from the circulation following the second and third infusion appeared to be short, 3.0 and 5.2 days respectively. After the fourth infusion the T1/2 increased to 14 days. The preinfusion ELISA titer at day 35, 56, and 77 posttransplantation decreased to significant ($p < 0.01$, Wilcoxon rank test) lower levels (median 760, range 250-1.300), as compared with the ELISA titers at day 7 and 14 posttransplantation (median 1.300, range 630-3900).

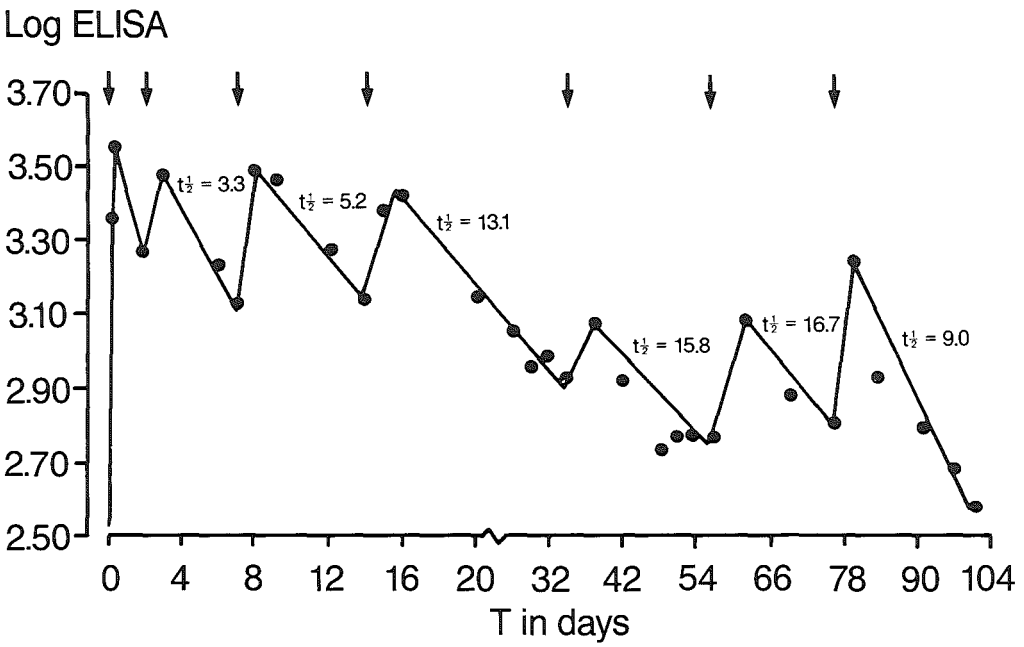

Fig. 5-1 **Log ELISA titers plotted as a function of time after transplantation in eight CMV seronegative patients. The data of each CMV-HIG infusion is marked with an arrow. The calculated half-life (hours) following each infusion is shown.**

3. CMV IgG ELISA and neutralizing antibody titers.

In 16 seronegative heart transplant recipients passive immunization induced geometrical mean preinfusion titers of 1.700-2.100 ELISA units during the first two posttransplant weeks. These levels remained at a median of 1.050 units during the following 3 months and rapidly decreased thereafter (fig. 5-2). Anti-CMV neutralizing geometrical mean preinfusion titers-1 were 16 during the first 2 weeks and 12 thereafter (fig. 5-2).

In 7/16 CMV seronegative recipients viremia was observed 35 (median, range 35-77) days after transplantation and 3/16 patients developed symptomatic CMV disease 26, 38 and 68 days respectively after transplantation.

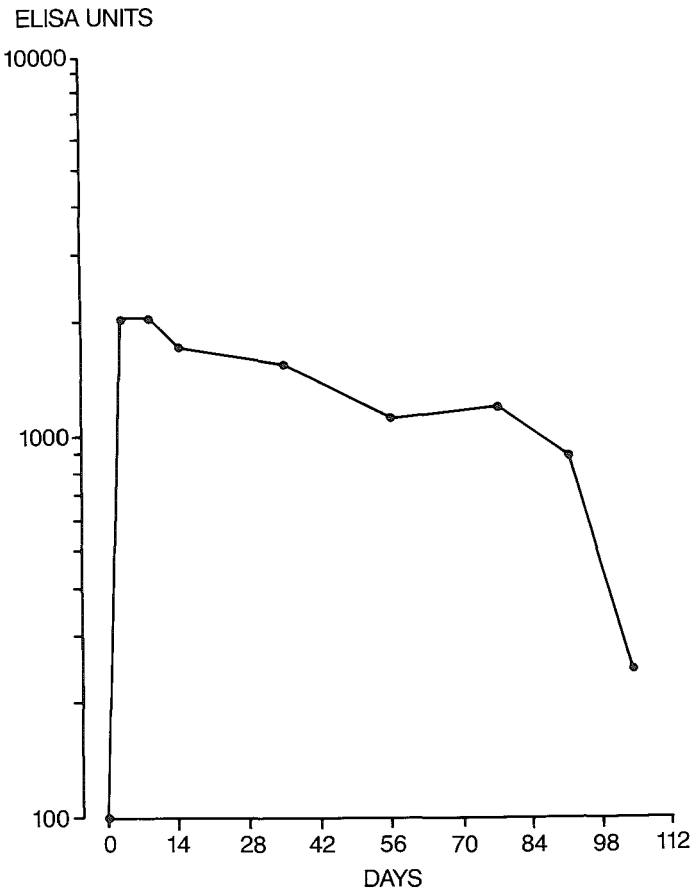

Fig. 5-2a **Anti-CMV IgG titers (ELISA) induced by passive immunization in 16 CMV seronegative (< 100 ELISA units) heart transplant recipients.**

The preinfusion ELISA IgG and NT in patients with and without viremia after transplantation were not significant different at any point. At the time of viremia the NT were not different compared to those at times without viremia. The 3 patients with CMV disease had no different preinfusion ELISA IgG and neutralizing antibody titers compared to the 13 patients without CMV disease.

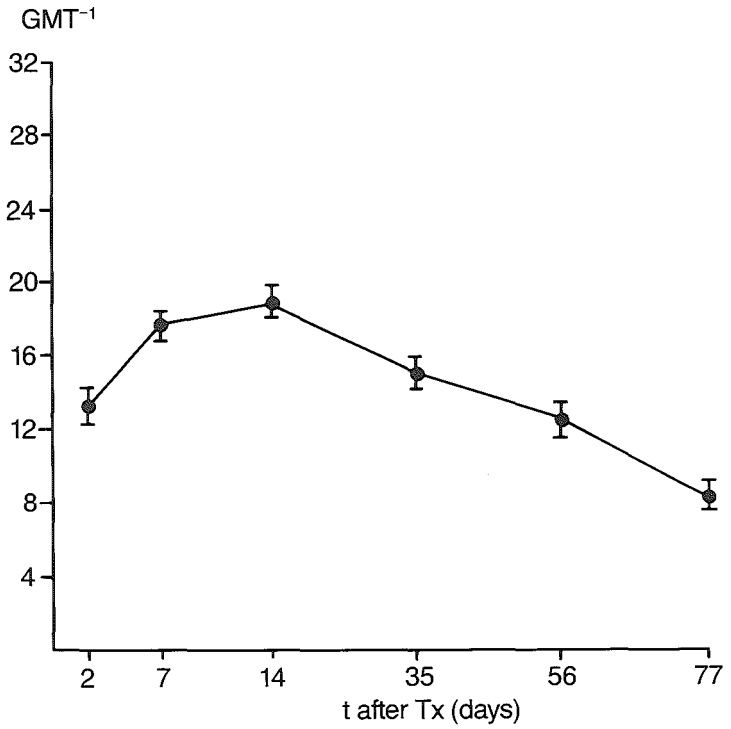

Fig. 5-2b **CMV neutralizing antibody titers induced by passive immunization in 16 CMV seronegative heart transplant recipients.**

4. Side effects.

A total number of 221 infusions of anti-CMV immunoglobulin were given in 32 patients. In two patients a rash necessitated discontinuation of the globulin treatment during the 6 th and 7 th infusion, but no hemodynamic side-effects were recorded in these two patients.

Blood pressure and heart rate (pacemaker rhythm) remained constant during and after the second globulin infusion (table 5-3 and fig. 5-3). Left atrium and central venous pressures increased from 8.9 to 10.0 mm Hg and from 10.4 to 11.1 mm Hg respectively (table 5-4 and fig. 5-4). During the other 189 infusions no hemodynamic side-effects were recorded.

	0	1	2	3	4	hrs
blood pressure syst.						
mean (mmHg)	127	126	127	129	125	
median	125	122	125	130	120	
range	90 - 160	90 - 166	95 - 165	95 - 162	95 - 165	
blood pressure diast.						
mean (mmHg)	79	77	79	81	81	
median	78	76	80	80	80	
range	55 - 120	50 -115	55 - 115	55 - 110	55 - 105	
heart rate (bpm)						
mean	106	105	105	105	105	
median	105	105	105	102	105	
range	87 - 124	88 - 120	88 - 120	84 - 120	84 - 120	

Table 5-3 Blood pressure and heart rate in 32 heart transplant recipients receiving passive immunization recorded during and after the second gift of the globulin preparation. The globulin preparation is infused in one hour.

bpm = beats per minute

	0	1	2	3	4	hrs
left atrium						
mean (mmHg)	9.2	10.0	10.1	10.3	10.4	
median	10	10	10	10	12	
range	3 - 18	4 - 18	3 - 18	3 - 20	3 - 21	
central venous						
mean (mmHg)	10.8	11.3	11.1	11.4	11.6	
median	11	12	12	12	13	
range	2 - 24	2 - 26	1 - 27	1 - 26	2 - 28	

Table 5-4 Left atrium and central venous pressures in 32 heart transplant recipients recorded during and after the second gift of an anti-CMV immunoglobulin preparation. The globulin preparation is infused in one hour.

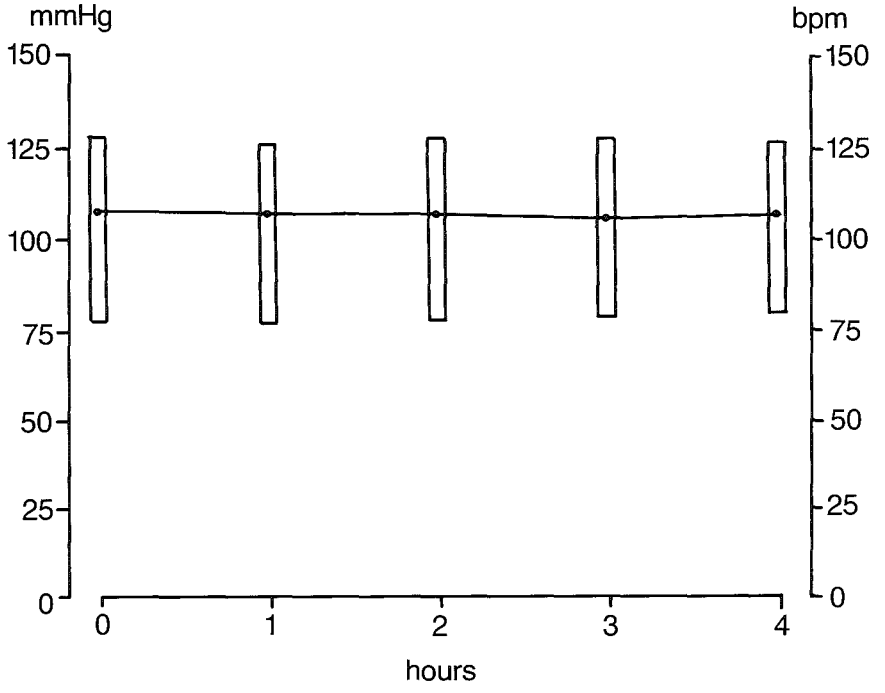

Fig. 5-3 **Systolic and diastolic bloodpressure and heart rate in 32 heart transplant recipients during and after the second gift of an anti-CMV immunoglobulin preparation.**

5. Virus transmission.

Before transplantation all recipients were HBsAg-negative and seronegative for HIV. Moreover all blood and organ donors were seronegative for HBsAg and HIV. After infusion of the immunoglobulin four patients showed seropositivity for HIV in the EIA. In three patients the seropositivity was already apparent after the first dose and became negative during the period of immunoprophylaxis. The other patient became seropositive after his fourth dose and was seronegative two weeks later. However, the Western Immunoblot did not show antibodies to HIV antigens in these patients. In none of them did clinical signs or symptoms of HIV infection become apparent in the follow-up period of 25-30 months after transplantation. No seropositivity for HBsAg could be detected after infusion of the globulin preparation. In two patients a rise in liver enzymes was noted after transplantation. In both these patients CMV was isolated during that period. In the other 30 patients no abnormalities in liver enzymes were observed during the median follow-up of 19 months.

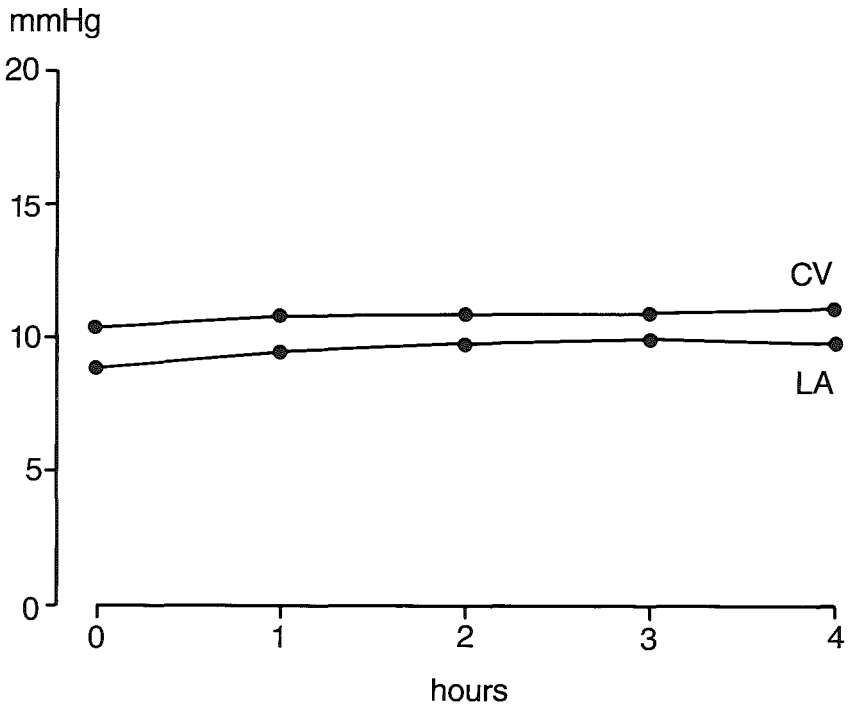

Fig. 5-4 **Filling pressures (LA = left atrium; CV = central venous) in 32 heart transplant recipients during and after the second gift of an anti-CMV immunoglobulin preparation.**

DISCUSSION

We treated all CMV seronegative recipients irrespective of donor serology with anti-CMV immunoglobulins from the day of transplantation. Arguments for this strategy were that the CMV serology of the donor is not always available at the time of transplantation and that CMV unscreened (although buffy coat poor) blood is used during open heart surgery. Moreover, in the majority (65 %) of the CMV seronegative heart transplant recipients rejections are treated in the first 3 months after transplantation and therefore these patients will become at high risk for CMV disease [26-27].

Passive immunization in the seronegative patients induced high anti-CMV ELISA titers (1.000-2.000 units) during the first 3 postoperative months, when the patients are at highest risk and anti-CMV neutralizing activity was observed as well during the same period. In contrast to the long half-life of other IgG preparations in other patient groups [23] this globulin preparation had a short half-life, four days during the first 14 days and increasing to 14 days during the following 90 days. This is in accordance with other studies in transplant recipients receiving different anti-CMV immunoglobulin

products [28, 29]. One explanation for this short half-life could be the catabolic effect of corticosteroids. Alternatively, complex formation with circulating virus could have led to antibody consumption, but unlikely during the first 2 weeks post transplant.

Based on the data from the literature it was expected to find an high incidence (82 %) of symptomatic CMV infection in CMV seronegative heart recipients from a CMV seropositive donor [1-3]. In the globulin treated patients we found a much lower (13 %) incidence of CMV disease. Only one patient had to be treated with DHPG and none of the seronegative patients died because of CMV infection. However, the incidence of CMV excretion was high (50 %) in these patients. The incidences of CMV isolation (42%) and related disease (11%) in the non globulin-treated CMV seropositive recipients were comparable with those in the globulin-treated CMV seronegative heart recipients from a seropositive donor. In the untreated CMV seronegative recipients from a CMV seropositive donor the incidence of CMV disease was 50 %. These observations suggest that passive immunization with anti-CMV immunoglobulin can prevent or mitigate symptomatic CMV infection and thus induces the same protection against CMV disease as natural acquired anti-CMV resistance. However, virus replication was not reduced by passive immunization and prophylaxis was only temporarily effective as can be learned from the case of the patient with symptomatic disease after the anti-CMV IgG titer had decreased to pretransplantation levels. The efficacy of prophylactic globulin treatment to reduce CMV disease, but not infection is in accordance with the studies in CMV seronegative renal transplant recipients from a seropositive donor [15-16].

In CMV seronegative heart recipients from a seronegative donor the incidence of isolation was much lower as compared with that in CMV seronegative recipients of a CMV seropositive allograft (6 % vs 50 %). In theory an incidence of 0 % was expected as transmission with the allograft is unlikely in the situation of a CMV seronegative allograft donor - recipient combination. However, we used CMV unscreened buffy-coat depleted blood during open heart surgery. Apparently, the use of buffy-coat depleted blood did not completely prevent CMV disease in all patients, not even when passive immunization was given.

It has been suggested that the therapeutical effect of anti-CMV immunoglobulin is associated with its neutralizing activity [22]. In a subgroup of patients both ELISA and CMV neutralizing antibody titers during immunoglobulin therapy were measured. No absolute protecting titer could be found. Heart transplant recipients without viremia had no statistically significant different higher neutralizing antibody or ELISA titers when compared to recipients with viremia. Moreover, immunoprophylaxis prevented disease and not viremia. Therefore, it is unlikely that the neutralizing activity of the globulin preparation alone is very important in the prevention of CMV disease. Other mechanisms of action could play a role. Grundy et al [30] suggested that immunoglobulin blocks CMV antigens on infected cells and mitigates the cellular immune response. As a consequence CMV associated symptoms are prevented or alleviated. Alternatively, antibody dependent cellular cytotoxicity (ADCC) can be induced by the immunoglobulin, causing destruction of virus infected cells. Another hypothesis is that the immunoglobulin binds to the Fc receptor of CMV infected cells, penetrates into the cell and neutralizes intracellular virus particles [31].

Apart from a slight increase in filling pressures, as was expected from the globulin with saline infusions, we have not detected an effect of the globulin preparation on the hemodynamic indices in the direct postoperative episode. In only 1 % of all transfusions minor side effects were observed. No severe anaphylactic reactions were observed.

Because of the use of huge plasma pools to prepare this anti-CMV immunoglobulin preparation, the risk for posttransfusion hepatitis is expected to be considerable, as compared to the reported incidence of non-A, non-B hepatitis of 10% in plasmatransfusions for clotting deficiencies [32]. However, in none of the recipients a case of non-A, non-B hepatitis was observed. In addition, virus transmission of HBV and HIV was not observed. This lack of transmission is probably due to the cold ethanol fractionation and ß-propionlactone treatment of the plasma pools [21]. ß-Propiolactone is an alkylating agent and therefore destroys DNA and RNA of viruses.

In conclusion, the anti-CMV immunoglobulin preparation used is a safe and well tolerated globulin, which can be used in immunosuppressed organ transplant recipients at risk for acquiring CMV infection. This study suggests that passive immunization with anti-CMV immunoglobulin prevents CMV disease, but not infection in CMV seronegative heart transplant recipients from a CMV seropositive donor. No correlation was found between anti-CMV IgG ELISA or NT reached and the incidence of CMV viremia or related disease.

One can argue that definite conclusions on the efficacy of passive immunization can only be drawn from a prospective, randomized, double-blind controlled study. However, the data from the present uncontrolled study in heart transplant recipients, the reports from controlled trials in kidney and bone marrow recipients [chapter 6, 15-19], may make such an approach questionable on ethical grounds in view of the reported high incidence of CMV disease [1-3].

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CHAPTER 6

PREVENTION OF CYTOMEGALOVIRUS RELATED DEATH BY PASSIVE IMMUNIZATION. A DOUBLE BLIND PLACEBO CONTROLLED STUDY IN KIDNEY TRANSPLANT RECIPIENTS TREATED FOR REJECTION.

INTRODUCTION

The incidence of Cytomegalovirus (CMV) disease is high in kidney transplant recipients treated for rejection [1]. Prevention or mitigation of this potentially lethal complication is therefore important. Recently it has been suggested that passive immunization with anti-CMV immunoglobulins can prevent CMV disease in CMV seronegative recipients of CMV seropositive kidney donors [2]. However, when all such patients irrespective of anti-rejection treatment are treated, a substantial number of them will receive unwarranted, costly and hazardous plasmaproducts. Moreover, CMV seropositive patients can also acquire serious CMV disease [3] and might benefit from immunoprophylaxis too.

In a double blind placebo controlled trial we have studied the value of passive immunization in both CMV seropositive and seronegative kidney transplant recipients treated for rejection.

PATIENTS AND METHODS

Study group

Between July 1985 and December 1987, 152 kidney transplantations were performed under cyclosporin A and low dose steroids. In 110 cases (72 %) no rejection was diagnosed and the incidence of CMV disease in these patients was low (6 %). The eligible group consisted of 42 patients, in whom a biopsy proved rejection necessitated therapy with rabbit anti-thymocyte globulin (RATG, National Institute for Public Health, Bilthoven, the Netherlands). Circulating T lymphocytes (Leu-4\CD 3) were kept between 75-150/mm^3 for 14 days. Cyclosporin A and steroids remained unchanged during anti rejection therapy. All were asked to participate in this study, which was approved by the Ethical Committee of our hospital. Forty patients agreed to participate and were randomized.

Preparations used

The anti-CMV immunoglobulin preparation was produced from cold ethanol precipitated large plasma pools with high titers of antibody against CMV (Cytotect^R, Biotest Pharma GmbH, Frankfurt, FRG). Cold sterilization was performed with ß propionolactone treatment [4]. The final preparation contained 100 mg protein/ml of which 95 % IgG. It had an anti-CMV IgG titer of 40.000 ELISA units/ml and a CMV neutralizing titer of 1:3000/ml [5]. As placebo a 20 % albumin solution was used

(Merieux, Lyon, France).During and after transplantation only buffy-coat depleted bloodtransfusions were given. Blooddonors were not regularly screened for CMV IgG antibodies.

Study design

Twenty patients received globulin and twenty patients received albumin. Both preparations were given iv over a period of one hour in a dose of 100 mg/kg body weight. The preparations were dissolved in 250 ml saline. Globulin/albumin infusions were given on the day of RATG treatment and on day 7, 14, 35, 56 and 77 thereafter. In CMV seronegative transplant recipients this dosage regimen resulted in median anti-CMV IgG titers of 1200 ELISA units during 3 months [6].

Virological studies

At the start of RATG therapy and before each infusion of globulin/albumin samples of urine, throatwash and peripheral blood leukocytes were collected for virus isolation. When indicated more specimens were obtained. The isolation of CMV was performed by a low speed centrifugation assay in combination with immunofluorescence by a monoclonal antibody against early antigen of CMV, as described in detail elsewhere [7]. All samples were also cultured on human embryonic lung fibroblasts; cultures were maintained for 6 weeks and screened for cytopathic changes. Specific anti-CMV immunofluorescence studies were done on all cultures. Before transplantation donor and recipient sera were screened for IgG antibody against CMV with an ELISA [8]. Titers <100 were considered to be CMV seronegative.

Clinical assessment

Symptomatic CMV infection was defined as illness without other explanations and with two of the following features : fever ($>38.5°C$) for at least 3 consecutive days; gastrointestinal-,lung- or central nervous system involvement; leukocytopenia ($<3.0x10^9/L$), thrombocytopenia ($<100 \times 10^9/L$) or elevated serum alanine or aspartate aminotransferase (>2.5 times the upper limit of normal). This viral syndrome had to be confirmed by concomitant isolation of CMV and/or pathognomonic features in autopsy or biopsy specimens. In case of potential lethal CMV disease therapy with DHPG (Syntex, United Kingdom) was instituted.

Statistical methods

For differences between the globulin and the placebo group the point estimate of the difference and its 95 % confidence interval (CI) are given. To test the

hypothesis that there was no difference between the globulin and placebo group, the chi-square test with Yates correction or the Student's-t test were used when appropriate. All results of tests of significance are reported as two tailed.

RESULTS

After randomization there was no statistically significant difference between the globulin and placebo treated groups for age, sex, CMV serostatus of donor and recipient or time between transplantation and rejection (table 1). One CMV seropositive patient died from a cerebral hemorrhage within 14 days after the start of globulin treatment. He developed no CMV infection in this period and was excluded from further analysis. No side effects were observed during or after the 223 infusions.

No differences for CMV infection and disease were found between the two seropositive groups and therefore they were combined. In table 2 the incidence of CMV isolation, viraemia, CMV disease and CMV related death is shown for all patients with stratification for CMV serostatus of the donor/acceptor combinations. CMV was isolated in none of the 8 seronegative allograft recipients of a CMV seronegative donor. There was no statistically significant difference between the incidence of CMV isolation from any site or of viraemia in the two subgroups at risk for primary and for

	GLOBULIN TREATMENT	PLACEBO TREATMENT
No of patients	20	20
Age (years)		
median	36	35
range	17-67	16-55
Sex		
male	13	12
female	7	8
Time trans-rej. (days)		
median	22	18
range	6-610	7-166
CMV serostatus Donor/Recipient		
Pos/Neg	5	4
Neg/Neg	3	5
Neg/Pos	5	5
Pos/Pos	7	6

Table 6-1 Characteristics of 40 renal transplant recipients treated for rejection with RATG

	GLOBULIN TREATMENT				PLACEBO TREATMENT			
CMV serostatus donor/recipient	**-/-**	**+/-**	**±/+**	**ALL**	**-/-**	**+/-**	**±/+**	**ALL**
No patients	3	5	11	19	5	4	11	20
Virus isolation	0	5	10	15	0	3	8	11
Viraemia	0	5	6	11	0	3	5	8
Disease	0	4	3	7	0	3	3	6
Lung involvement	0	2	0	2	0	3	0	3
Death	0	0	0	0	0	3	1	4

Table 6-2 **The incidence of CMV isolation and disease in globulin/placebo treated recipients stratified for CMV serostatus of the donor/recipient combination.**
-/- : seronegative donor/seronegative recipient
+/- : seropositive donor/seronegative recipient
±/+ : seropositive or seronegative donor/seropositive recipient

secondary CMV infection. However, CMV related disease was more frequently diagnosed in the 9 seronegative recipients at risk as compared with the 22 seropositive recipients (78 vs 27 %, difference 51 %, 95 % CI 12 to 89 %, $p < 0.02$) and cases of lung involvement were only observed in the seronegative recipients (difference 56 %, 95 % CI 27 to 84 %, $p < 0.01$). The 8 CMV seronegative recipients of seronegative allograft donors were not challenged with CMV and therefore not at risk for CMV infection.

Between the globulin (n=16) and placebo (n=15) treated groups at risk no statistically difference in incidence of CMV isolation from any site was observed (94 % vs 73 %). Viraemia was detected in 11/16 (69 %) globulin treated patients and in 8/15 (53 %) placebo treated patients (difference not statistically significant). Three patients of the globulin group and none of the placebo group were already viraemic at the first day of r-ATG treatment. Seven patients in the globulin group and 6 patients in the placebo group developed CMV disease (difference not statistically significant). CMV disease was diagnosed at day 7 (median, range 6-81) of globulin treatment and at day 11 (median, range 5-90) of placebo treatment. None of the 16 globulin treated patients died from CMV infection in contrast to 4/14 placebo treated patients (difference 27 %, 95 % CI 3 to 50 %, n.s.), despite intensive treatment including DHPG. In patients with virus isolation, viraemia or viral disease the difference in CMV related mortality was statistically significant, as is shown in fig. 6-1. CMV related death occurred in 4/8 placebo treated patients with viraemia (difference 50 %, 95 % CI 13 to 87 %, $p < 0.05$). In patients with CMV disease this difference was 67 % (95 % CI 16 to 117 %, $p < 0.05$).

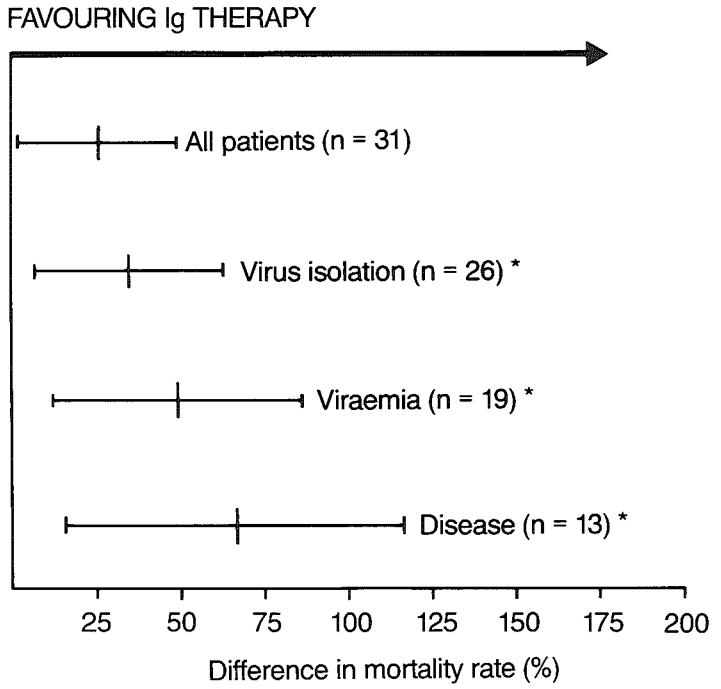

Fig. 6-1 **Anti-CMV immunoglobuline therapy versus placebo treatment in kidney transplant recipients treated with RATG for rejection.**
Effect on CMV related mortality in all patients at risk, in patients with virus isolation, in patients with viraemia and in those with CMV disease: approximate 95 confidence intervals for difference in mortality rates.
*** $p < 0.05$**
n = number of patients.

DISCUSSION

Passive immunization completely prevented fatal CMV disease in kidney transplant recipients treated for rejection, although it did not reduce the number of patients with CMV isolation, viraemia or CMV disease. None of the 8 CMV seronegative recipients of a seronegative allograft donor acquired CMV infection. Apparently these patients are not at risk although they all received unscreened bloodtransfusions. CMV disease was more frequently observed and was more severe in the seronegative patients at risk (78 %) than in the seropositive recipients (27 %), but the incidences of CMV isolation or of viraemia in these groups were comparable. In the 31 patients at risk, the incidence of CMV infection and disease was high: 87 % CMV isolation, 63 % viraemia, and 43 % disease. No differences were observed between the globulin and placebo treated patients for virus isolation or virus related disease. However, CMV related death only occurred in the control group. In the open labelled multicenter trial reported by Snydman et al [2] also no effect of

immunoprophylaxis on the incidence of CMV infection was demonstrated. However, at variance with the present study, a reduction in severe CMV disease but not in CMV related mortality was found. An explanation for this difference could be the moment of passive immunization. Snydman et al started on the day of transplantation and we at the moment a biopsy proved rejection necessitated RATG treatment, because we felt that the majority of our patients were not at risk for CMV disease. Indeed 110 of our 152 patients (72 %) showed no signs of rejection and their incidence of CMV disease was low (6 %). Another explanation for the discrepancy could be the difference in entry criteria of the two studies. We included all patients receiving RATG, but irrespective of their CMV serostatus. Snydman et al treated only seronegative allograft recipients of a seropositive donor, but irrespective of rejection treatment. In both studies the difference between the globulin and placebo treated groups was mainly due to an effect in the seronegative patients treated with ATG.

We were not able to show efficacy in the CMV seropositive patients treated for rejection and therefore we cannot advise the prophylactic use of anti-CMV immunoglobulin in these patients. We agree with Snydman et al that CMV seronegative candidates receiving kidney transplants from CMV seropositive donors should be considered for globulin prophylaxis. However, not all of these patients run a high risk for acquiring CMV disease, as the incidence is relatively low in patients not treated for rejection. As a consequence, some of them will receive unnecessary costly and potential hazardous globulin therapy. We showed that passive immunization is still effective when initiated at the time the patient becomes at risk by RATG treatment. CMV seronegative transplant recipients with a CMV seropositive donor can be successfully protected from a fatal CMV disease when passive immunization is started at the time of anti rejection therapy.

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CHAPTER 7

SUMMARY

Although the introduction of Cyclosporin (CsA) as the main immunosuppressive agent seems to have influenced the incidence and severity of CMV disease in a positive way, the reported incidence of clinical overt CMV infection is still 2 to 23 % and 1 to 3 % of the transplant recipients die from CMV infection. It is therefore obvious that this virus remains a major pathogen after organ transplantation. This thesis describes two methods for rapid diagnosis of CMV infection, risk factors for acquiring symptomatic CMV infection after renal transplantation and possibilities for the prevention of CMV infection in renal and heart transplant recipients.

DIAGNOSIS OF CMV INFECTION

Rapid, sensitive and specific assays for the early diagnosis of active CMV infection are imperative in immunosuppressed transplant patients. When CMV disease is diagnosed, reduction of immunosuppressive therapy will markedly decrease morbidity and mortality without affecting graft survival. Moreover, antiviral agents as e.g. DHPG (Ganciclovir) have been shown to be effective in organ transplant recipients with severe CMV infections. However, diagnosis either by virus isolation or by serological methods is hampered by lack of sensitivity, specificity and speed. In chapter 3 the results from a centrifugation assay are compared with the results from the conventional tissue culture system. The centrifugation assay consists of low-speed centrifugation of the specimen on human embryonic lung cells, followed by detection of Cytomegalovirus early antigen using a monoclonal antibody in an immunofluorescence technique. 161 specimens obtained from 52 patients were studied; from 14 patients CMV was isolated in at least one specimen (in total 28 specimens). The centrifugation assay led to positive results within 24 to 48 hours, whereas the cell culture took an average of 16.5 days to develop the typical cytopathic changes. No cross-reactions between the monoclonal antibody used and other viruses present (Herpes simplex and Adenovirus) were observed. Considering the conventional cell culture as the golden standard, the sensitivity of the centrifugation assay was 91 %, and the specificity 97 %. False negative results of the centrifugation assay were all from buffy coat cells.

T cell subset monitoring can be of predictive value in transplant patients with an imminent virus infection, as is described in chapter 3. The effect of Cytomegalovirus disease on mononuclear subpopulations of 49 renal patients treated with cyclosporin and prednisone were studied. Clinical overt CMV infection developed in 8/21 patients treated for rejection with rabbit antithymocyte globulin. They all showed true inversions of the CD4/CD8 ratio. A reduction of CD4 positive cells and increase in CD8 positive cells preceded clinical symptoms of CMV disease by one week. None of the 26 patients without RATG anti-rejection treatment developed CMV disease and in only three of them an inversion of CD4/CD8 ratio was found.

HIGH RISK GROUPS

In immunocompetent individuals primary CMV infection induces cellular and humoral immune responses leading to immunity as reflected by seropositivity without clinical illness. In contrast primary CMV infections, reactivations and reinfections frequently result in symptomatic disease in immunocompromized organ transplant recipients. CMV seronegative allograft recipients can acquire the virus from an allograft of a CMV seropositive donor, as was first described by Ho et al (1975) and Betts et al (1975). Many epidemiological studies on this route of transmission have confirmed these early observations. In chapter 4 the results of such a study are described. In two Dutch transplantation centers the incidence of CMV infection and disease in 73 renal transplant recipients were prospectively studied according to the CMV serostatus of organ and blooddonor/acceptor combinations. Primary CMV infection occurred in 8/19 (42 %) CMV seronegative patients. All eight had received a graft from a seropositive donor. CMV associated symptoms were observed in six and CMV related death in two of them. In contrast, no CMV infection was observed in CMV seronegative recipients of a kidney from a seronegative donor. The incidence of primary and secondary CMV infection in patients at risk were comparable: 67 vs. 54 %. However, the incidence and severity of CMV disease was significantly higher after primary infection (50 vs 17 % X^2, $P < 0.05$). Primary CMV infection also had a detrimental effect on graft survival. The seropositive donor-seronegative acceptor combination had a three year graft survival of 41 %, while the other three donor/acceptor combinations showed a three year graft survival of 72 %. The use of high doses corticosteroids and especially the use of antilymphocyte globulin (ALG) preparations given for rejection are associated with an increased incidence and severity of CMV infection after renal transplantation, as is described in chapter 4. The overall incidence of CMV disease depends on the percentage of patients treated with ALG in a given population. Obviously it also depends on the number of CMV seronegative recipients of an allograft from a seropositive donor. When this subgroup is treated with ALG, it becomes double at risk and it is not surprising that 54-100 % of these patients acquire CMV disease with fatality rates up to 28 %.

PREVENTION OF CMV DISEASE

Possible methods to prevent virus diseases include avoidance of virus transmission, active or passive immunization, and prophylactic antiviral therapy. Avoidance of CMV transmission in organ transplantation is a distinct possibility. When organs from a CMV seronegative donors are transplanted into seronegative recipients and when leukocyte - depleted blood are given, the incidence of primary CMV infection is low, as described in chapter 4. Such a policy is not unusual in heart/lung, liver and bone-marrow transplantation programs, probably because the incidence of fatal CMV disease was much higher than after kidney transplantation. Nevertheless, although the overall percentage of kidney transplant recipients who die from CMV disease is lower. (1-3 %), the sheer number of kidney transplantations performed (2521 within the Eurotransplant Organization in the first 10 months of 1989) will likely result in a substantial number of patients dying from CMV infection. Reserving CMV negative donor kidneys for CMV negative recipients certainly will save lives but may

lead to a prolonged waiting time for a transplant because of shortage of CMV negative donors. In a large organ sharing organisation this problem can be limited without significant concessions to HLA matching. However, this strategy is unacceptable for critically ill heart and liver transplant candidates, as it does prolong the waiting time on the list.

Passive immunization with anti-CMV immunoglobulins has been the subject of several studies. Most of these were uncontrolled, controlled with placebo preparation containing anti-CMV immunoglobulins, or open labeled studies. Comparison of these studies is difficult because of differences in the preparations used, in dosage schemes and in the methods used for the diagnosis of CMV infection. In chapter 5 and 6 two studies on passive immunization in renal and heart transplant recipients are described. In a double-blind placebo controlled trial the value of passive immunization in both CMV seropositive and seronegative kidney transplant recipients treated for rejection with ATG was studied. The justification for the eligible patient group was that only patients treated for rejection run a high risk for acquiring CMV infection.

When all patients, irrespective of anti-rejection treatment, are passively immunized, a substantial number of them will receive unwarranted, costly and potentially hazardous plasma products. The reason to include CMV seropositive patients too comes from the observation that these patients can also acquire serious CMV disease and might therefore also benefit from immunoprophylaxis. In 42/152 consecutive kidney transplant recipients anti-rejection therapy was indicated. Two patients refused the protocol and 40 patients were included in the study. Globulin treatment, when started on the day of anti-rejection therapy did not influence the incidence of virus isolation, viremia or disease in the kidney transplant recipients. However, passive immunization completely prevented CMV related death. This beneficial effect was only observed in the seronegative recipients of a kidney from a seropositive donor.

After heart transplantation all CMV seronegative recipients irrespective of donor serology or antirejection treatment received prophylactic anti-CMV immunoglobulins from the day of transplantation. Arguments for this strategy included that serology of the heart donor is not always available and that unscreened (although buffy coat poor) blood is used during open-heart surgery in our center. Moreover in the majority of heart transplant patients (70 %) rejections are diagnosed in the first 3 months after transplantation and therefore these patients may become double at risk for CMV disease, when anti-rejection treatment is instituted. In an open study 32 CMV seronegative heart transplant recipients received immunoglobulin during the first 90 days after transplantation. Thirty-one seropositive recipients served as controls. Passive immunization in the seronegative patients induced high anti-CMV ELISA titers (1.000 -2.000 units) during the first 3 months after transplantation. The half life of the globulin preparation used was rather short, ranging from 4 days during the first 14 days of transplantation and increasing to 14 days thereafter. During the period of passive immunization anti-CMV neutralizing activity was observed in all patients studied. No differences in antibody titers reached during the immunoprophylactic period could be demonstrated between patients with and without viremia after transplantation. In the double seronegative donor/acceptor combination a low incidence of CMV infection (6 %) was observed. Probably passive immunization is not indicated for this subgroup of patients, because the risk of virus transmission with the donorheart or with blood transfusions is, although theoretically possible, practically nil. High CMV infection rates were found both in the non-globulin treated seropositive patients (39 %) and in the globulin-treated seronegative patients at risk (50 %). This

indicates that the presence of anti-CMV antibodies, either natural acquired or passively administered, is not able to prevent CMV replication. However, a low incidence of CMV disease (13 %) was observed in the globulin-treated patients despite a high infection rate. This suggests that anti-CMV antibodies prevent the development of CMV disease in patients with infection. No hemodynamic side-effects related to the globulin infusion were observed. Moreover, in none of the patients transmission of viruses, as e.g. Human Immunodeficiency virus, Hepatitis non A, non B and Hepatitis B virus through these plasmaproducts could be demonstrated.

In conclusion, these studies indicate that passive immunization against CMV is a safe procedure that prevents fatal CMV disease in seronegative RATG treated kidney transplant recipients in case of a seropositive donor. In seronegative heart transplant recipients of a heart from a seropositive donor, passive immunization from the day of transplantation induces the same protection against CMV disease as natural acquired anti-CMV immunity.

SAMENVATTING

Niertransplantatie is de voorkeursbehandeling bij patienten met een terminale nierinsufficientie. Transplantatie van andere organen, zoals hart en lever, is pas sedert het gebruik van het immunosuppresieve middel cyclosporine A (CsA) algemeen geaccepteerd als behandeling van patienten met eindstadium hart - of leverfalen. De immunosuppressieve behandeling, die noodzakelijk is om afstotingsreacties te voorkomen en te behandelen, maakt de patient na orgaantransplantatie vatbaarder voor infecties. Met name virusinfecties spelen een belangrijke rol na orgaantransplantatie. Eén van deze virussen is het Cytomegalievirus (CMV). Dit virus werd waarschijnlijk voor het eerst beschreven in 1904 door Jesionek, die vergrote cellen met intranucleaire insluitlichaampjes aantrof in obductiemateriaal van pasgeboren kinderen. De laatste 20 jaren is de kennis over dit DNA virus, dat tot de familie van de herpesvirussen behoort, enorm toegenomen. Uit epidemiologische studies bleek dat ongeveer 50 % van de West-Europese bevolking in de eerste 30 jaren van zijn leven besmet wordt met dit virus. Echter het merendeel van deze infecties verloopt zonder symptomen. Het virus blijft wel latent aanwezig in het lichaam. Onderdrukking van het afweerapparaat, bijvoorbeeld als gevolg van de immunosuppressieve behandeling na orgaantransplantatie, veroorzaakt een toename van symptomatische CMV infecties, waarvan sommige zelfs met dodelijke afloop. Deze infecties kunnen zowel primair als secondair (reactivatie van het latent aanwezige virus) zijn.

DIAGNOSTIEK VAN CMV INFECTIES

De behandeling van symptomatische CMV infecties na orgaantransplantatie is gebaseerd op enerzijds vermindering van de immunosuppressieve therapie en anderzijds het geven van antivirale geneesmiddelen, zoals bijvoorbeeld Ganciclovir. Een snelle, betrouwbare diagnostiek van CMV infecties is derhalve van groot belang. De diagnostiek was tot recent gebaseerd op virusisolatie in een celkweek en/of 4-voudige antilichaam titer stijging. Beide methoden zijn langzaam en / of weinig gevoelig. In hoofdstuk 3 van dit proefschrift worden de resultaten van een nieuwe diagnostische methode vergeleken met die van de klassieke celkweek. Materiaal (urine, keelspoelsel, bloed) afkomstig van een patient, die verdacht wordt van een CMV infectie, werd geënt op een cellaag van menselijke embryonale long cellen en gecentrifugeerd gedurende 1 uur op een lage snelheid. Aansluitend werd het kweekmateriaal gedurende 24 en 48 uur geincubeerd bij 36° C. Hierna volgde kleuring met een muizen monoclonaal antilichaam gericht tegen een vroeg antigeen van CMV. Aan het muizen monoclonaal antilichaam werd fluorescerend isothiocyanaat-gebonden anti-muis IgG toegevoegd. Hierna volgde een beoordeling onder de miscroscoop op fluorescentie in de kern van de cel. Dit werd als een positieve CMV isolatie geduid. Bij de klassieke celkweek werd 2 maal per week gekeken naar het specifieke cytopathogene effect, dat CMV veroorzaakt in een kweek van menselijke embryonale longcellen. Beide methoden werden vergeleken bij 161 materialen afkomstig van 52 patienten. Uit 28 materialen afkomstig van 14 patienten

werd CMV geisoleerd. De centrifugatie methode was gemiddeld 14 dagen eerder positief dan de klassieke kweekmethode. De gevoeligheid en de specificiteit van de centrifugatie methode was groot, respectievelijk 91 en 97 %. Alleen de isolatie van CMV uit bloed was minder gevoelig met de centrifugatie methode in vergelijking met de celkweek. Slechts 2 van de 5 bloedmonsters die positief waren in de celkweek bleken eveneens positief in de centrifugatie methode.

Een meer indirecte methode om CMV infectie te detecteren is de bepaling van de subpopulaties van T-cellen in het perifere bloed, zoals beschreven in hoofdstuk 3. Bij 49 niertransplantatie patienten werd het effect van symptomatische CMV infectie op de T-cel subpopulaties bestudeerd. Bij 8 patienten was er sprake van een ziektebeeld, veroorzaakt door CMV. Alle 8 patienten waren behandeld voor een afstoting met konijnen-antithymocyten globuline. Bij alle patienten was er sprake van een omkering van de CD4 / CD8 verhouding, als gevolg van een toename van de CD8 positieve cellen en een geringe afname van de CD4 positive cellen. Deze verandering trad al op in de week voor het begin van de klinische symptomen. Geen van de 26 patienten zonder afstoting na transplantatie ontwikkelde een symptomatische CMV infectie. Bij 3 van deze patienten was er wel sprake van een omkering van de CD4/CD8 ratio, mogelijk gerelateerd aan andere virale infecties.

RISICO GROEPEN

Het risico op het krijgen van een symptomatische CMV infectie na orgaantransplantatie is niet voor alle patienten gelijk, zoals aangetoond wordt in hoofdstuk 4. Transmissie van CMV door middel van een orgaantransplantaat werd voor de eerst maal beschreven in 1975. Sedertdien hebben vele epidemiologische studies dit bevestigd. Echter, het merendeel van deze studies was afkomstig uit de Verenigde Staten en Groot-Britannië, waar de prevalentie van CMV infectie verschilt met die in Nederland. Verder bestudeerden deze studies niet tegelijkertijd het risico van transmissie van CMV door bloedtransfusies rondom de transplantatie. Dit was de aanleiding tot het verrichten van een studie in twee Nederlandse tranplantatie centra over de incidentie van CMV infectie in 73 niertransplantatie patienten en de relatie met de CMV serologische status van bloed-en orgaan donor en ontvanger.

Primaire CMV infectie werd gezien in 8/19 (42%) CMV seronegatieve ontvangers. Alle 8 patienten kregen een orgaan afkomstig van een CMV seropositieve donor. Bij 6 van deze patienten was er sprake van een CMV gerelateerd ziektebeeld en 2 patienten overleden ten gevolge van de CMV infectie. In tegenstelling tot deze 8 patienten ontwikkelde niemand van de CMV seronegatieve ontvangers van een transplantaat afkomstig van een CMV seronegatieve donor CMV infectie, ondanks het feit dan 75% van de patienten bloed kreeg afkomstig van CMV seropositieve donoren. De incidentie van secundaire CMV infectie was vergelijkbaar met die van primaire infectie (54 vs 67%). Echter, de incidentie en ernst van de symptomatische CMV infectie was beduidend hoger in CMV seronegative ontvangers in vergelijking met de CMV seropositieve ontvangers (50 vs 17%). De primaire CMV infectie had ook een nadelige invloed op de transplantaatfunctie. Drie jaar na transplantatie functioneerde slechts 41 % van de nieren in tegenstelling tot 72 % in de patienten zonder CMV infectie.

De incidentie symptomatische CMV infecties bij transplantatiepatienten, die behandeld werden voor een afstoting met konijnen antithymocyten globuline was veel

hoger dan bij patienten, die geen afstotingsbehandeling kregen. Acht van de 23 met konijnen ATG behandelde niertransplantatiepatienten ontwikkelden een symptomatische CMV infectie 3-5 weken na de eerste gift ATG in tegenstelling tot geen van de 26 patienten zonder ATG behandeling.

PREVENTIE VAN CMV INFECTIES

De behandeling van een symptomatische CMV infectie bestaat, zoals al eerder is aangegeven uit immuunreductie in combinatie met antivirale therapie. Echter de toxiciteit van Ganciclovir, het ontstaan van resistentie tegen dit geneesmiddel en het falen van de antivirale therapie in sommige patienten met ernstige CMV infecties onderstreept nog eens een stelregel in de geneeskunde :"voorkomen is beter dan genezen". In hoofdstuk 4 wordt beschreven dat CMV seronegatieve ontvangers van een orgaan afkomstig van een CMV seronegatieve donor geen CMV infectie krijgen in tegenstelling tot de CMV seronegatieve ontvangers van een orgaan afkomstig van een CMV seropositieve donor. Door middel van selectie van CMV seronegatieve organen voor deze subgroep van transplantatiepatienten kan CMV infectie worden voorkomen. Echter deze strategie is niet altijd uitvoerbaar en kan de wachttijd voor transplantatie verlengen. Dit is veelal niet acceptabel voor ernstig zieke hart- en levertransplantatie kandidaten.

Passieve immunizatie met anti-CMV immunoglobuline preparaten zou symptomatische CMV infectie na transplantatie kunnen voorkomen. In hoofdstuk 5 wordt het effect van passieve immunizatie in 32 CMV seronegatieve hart transplantatie patienten bestudeerd. De passieve immunizatie werd gegeven gedurende de eerste 3 maanden na transplantatie en resulteerde in hoge anti-CMV ELISA IgG titers. In één van de 16 CMV seronegatieve ontvangers van een CMV seronegatief hart werd een symptomatische CMV infectie gevonden. In de CMV seronegatieve ontvangers van een CMV seropositief hart werd een hogere incidentie CMV infecties waargenomen. Acht van de zestien patienten ontwikkelden een CMV infectie na transplantatie. Echter, in tegenstelling tot hetgeen verwacht was op basis van de gegevens uit de literatuur, werd slechts bij 2 patienten (13%) een symptomatische CMV infectie vastgesteld. Deze incidentie is vergelijkbaar met die in de CMV seropositieve hart patient en suggereert dat passieve immunizatie in CMV seronegatieve ontvangers van een CMV seropostief orgaan dezelfde bescherming tegen symptomatische CMV infectie biedt als de natuurlijk verworven afweer. De resultaten van deze open studie worden bevestigd door de dubbel-blinde, placebo gecontrolleerde studie in niertransplantatie patienten, zoals beschreven in hoofdstuk 6.

In deze studie werden zowel CMV seropositieve als CMV seronegatieve patienten, die een afstotingsbehandeling met konijnen-ATG kregen, bestudeerd. Vanaf het moment van afstotingsbehandeling werden deze patienten passief geimmmuniseerd gedurende 3 maanden. De controle groep kreeg een albumine-oplossing. Veertig patienten werden opgenomen in deze studie. Passieve immunizatie voorkwam het optreden van CMV infecties niet, noch in de CMV seronegatieve, noch in de CMV seropositieve niertransplantatie patienten. Ook het aantal patienten met een symptomatische CMV infectie was in beide groepen gelijk. Echter, in de met anti-CMV immunoglobuline behandelde groep overleed niemand aan de CMV infectie in tegenstelling tot 4/15 patienten in de placebo behandelde groep. Dit verschil was

statistisch significant en werd bepaald door de resultaten in de groep van CMV seronegatieve ontvangers van een CMV seropositief orgaan.

Samenvattend kan gesteld worden dat CMV seronegatieve ontvangers van een CMV seropositief orgaan een verhoogd risico hebben op het krijgen van een symtomatische CMV infectie na transplantatie. Dit risico neemt nog eens toe, wanneer deze patienten een of meerdere afstotingsbehandelingen krijgen. Passieve immunizatie met anti-CMV immunoglobuline kan de incidentie en de ernst van symptomatische CMV infectie positief beïnvloeden.

This thesis is based on the following publications of the author:

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NAWOORD

Orgaan transplantatie vereist een multidisciplinaire aanpak. Dit proefschrift is daar een voorbeeld van. Vele afdelingen, waaronder de virologie, cardiologie, thoraxchirurgie, algemene heelkunde en inwendige geneeskunde, hebben geparticipeerd in de studies over het Cytomegalievirus na orgaan transplantatie. Ik ben dan ook vele mensen mijn dank verschuldigd. Enkelen wil ik met name noemen.

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Dit proefschrift is opgedragen aan mijn twee lieve dochters Esther en Thérèse.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 13 maart 1955 te Hoogeveen. Na het behalen van het diploma Gymnasium ß aan het christelijk lyceum "Overvoorde" te Rijswijk in 1973 volgde hij de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Het doctoraal examen werd afgelegd in 1978 en het artsexamen op 21 december 1979. Aansluitend werd begonen met de opleiding tot internist op de afdeling Interne Geneeskunde I, Academisch Ziekenhuis Rotterdam-Dijkzigt te Rotterdam, opleider Prof. dr J. Gerbrandy en later Prof. dr M.A.D.H. Schalekamp. Inschrijving in het specialisten register volgde op 31 december 1984. Hierna was hij tot september 1988 werkzaam als internist op de afdeling Heelkunde van het Academisch Ziekenhuis Rotterdam-Dijkzigt. Vanaf die tijd is hij verbonden aan de afdeling Inwendige Geneeskunde II (hoofd Prof. J.H.P. Wilson) van hetzelfde ziekenhuis en houdt zich speciaal bezig met levertransplantatie. Hij is getrouwd met Ellen Bronk en heeft twee dochters Esther en Thérèse.