9

General discussion

CONTENTS

HERPES SIMPLEX VIRUS TYPE-1

Role in oral ulcerations

Based on our knowledge of the pathogenesis of HSV-1 infections a causative role for HSV-1 in oral ulcerations in hematopoietic stem cell transplant (HSCT) recipients is very plausible. However, the contribution of herpesviruses to oral ulcerations in this setting has never been studied systematically, by sampling all cases regardless of the presence of ulcerations. The multifactorial etiology of oral ulcerations during and after HSCT and the fact that asymptomatic oral shedding of serveral viruses has been described during immunesuppression warrant this type of study, as described in chapter three.

Hence, the relative contribution of chemoradiation and different herpesvirus infections to oral ulceration after HSCT was studied. In the study in chapter three the presence of HSV-1 was a significant positive predictor for both ulcerative mucositis and ulcerations of the keratinized mucosa. Truly asymptomatic oral HSV-1 shedding occurred rarely. Since conditioning regimen and donortype were no predictors of oral ulcerations and since the rate of HSV-1 shedding was high, the relative contribution of HSV-1 to oral ulcerations after HSCT should be considered large. These findings support the use of antiviral prophylaxis with (val)aciclovir ((v)ACV) in this patient group.[1-6] Without prophylaxis, prompt administration of antivirals upon the development of oral ulcerations awaiting viral diagnostics should certainly be considered.

Interestingly, also the presence of EBV in oral washing samples was an independent predictor of oral ulcerations on the keratinized mucosa, but not of ulcerative mucositis. The pathogenetic role of EBV in oral ulcerations is not well-known. EBV has clearly been associated with oral hairy leukoplakia in various categories of immunocompromised patients.[7] EBV-associated oral ulcerations have also sporadically been described.[8,9] However, a causative role of EBV in oral ulcerations shortly after HSCT has not been proven by our study nor by others. Its presence may rather be the consequence of increased shedding in the presence of (HSV-1-induced) ulcers rather than being the cause of ulceration. Prophylaxis of HSV-1 with (v)ACV may decrease oral shedding of EBV, because EBV is susceptible to ACV during lytic infection.[10-15] Still, it is unclear whether EBV in oral ulcerations represents lytic infection, comparable to mononucleosis and oral hairy leukoplakia, or expansion of latently EBV infected cells, comparable to EBV related lymphoproliferative disorders, and the response to antiviral prophylaxis is therefore uncertain.[16-21] Certainly, the possible role of EBV in oral lesions in HSCT recipients merits further study.

Antiviral resistance

In clinical diagnostics, viral sequence analysis to detect resistance-associated mutations has the advantages of speed and technical ease. Nevertheless, as shown previously[22;23] and in chapter two and three, mutations of unknown significance are commonly found in HSV-1 clinical isolates. This demonstrates the need for a phenotypical susceptibility test to establish the significance of such mutations. The classical plaque reduction assay (PRA) can be used for this purpose, but because of its long assay time, a faster assay is needed.

In the study described in chapter two a faster and more easily applied protocol for phenotypical susceptibility testing of HSV-1 was developed. Results of the DNA reduction assay (DRA) compared very well to results obtained by genotypic tests and by PRA. Moreover, low level resistance to ACV and FOS was more accurately detected by DRA than by PRA. However, low-level resistance or intermediate susceptibility is not defined in the CLSI protocol[24] or by breakpoints suggested previously[25] and it remains to be investigated if infections with such isolates should be treated differently from high-level resistant isolates.

A two-step approach is likely to be most practical in the clinical setting, starting with target gene sequencing of, preferably, a pre- and on-treatment sample and subsequent phenotypical confirmation of resistance if mutations of unclear significance are encountered. As described in chapter two, DRA was successfully applied to confirm susceptibility to ACV in an isolate with a previously not well characterized mutation.[22;26] Also, in chapter three DRA demonstrated ACV resistance in an isolate with a novel mutation. This demonstrates the utility of a two-step approach for HSV-1 resistance analysis.

Treatment failure and antiviral resistance

In chapter three, HSCT recipients were systematically monitored for persistent oral replication of HSV-1. Oral shedding after a standard course of antiviral therapy for five days occurred in 43% of the patients and was due to resistance in 18% of treated patients. Of course, sensitive detection of HSV-1 DNA by real-time PCR after ulcerations have (almost) healed may account for part of the persistence. However, given the very short half-life of DNA,[27-31] such detection must represent at least recent viral replication rather than being a remnant of past replication.

The retrospective analysis of resistance in our study hampers establishing the clinical significance of the infections with resistant HSV-1. Probably, resistant isolates have reduced fitness and virulence[32;33] or are rapidly cleared as soon as immunological recov-

ery occurs. Nevertheless, a protracted course with severe ulceration occurred in several patients in our study and as patients have reported that oral mucositis was the single most debilitating side effect of HSCT conditioning,[34] its possible clinical relevance should not be ignored. Therefore, persistent oral ulcerations despite antiviral treatment demand viral diagnostics and antiviral resistance testing, to optimize treatment, both for patients with and without resistant HSV-1.

VARICELLA-ZOSTER VIRUS

Antiviral resistance

Because VZV is a rather slow growing and highly cell-associated virus and is often present in samples from which it cannot be cultured (plasma, CSF), resistance analysis is usually performed by sequence analysis of the genes involved in antiviral resistance.[35-40] As described previously[41] compartmentalization of antiviral resistance in sanctuary sites such as CSF and eye was found in a relevant proportion of the patients with resistant virus in our study in chapter four. In addition, amplification of full length viral genes from CSF and eye samples was often problematic necessitating us to adapt the protocol for such samples (using smaller amplicons). This may be related to the viral loads in the samples, but may also be due to the presence of fragmented viral DNA.[42]

Using sequence analysis as a resistance assay, the significance of mutations that have not been described before, which were found in half of the patients with resistant virus in chapter four, cannot be determined with certainty. Comparing sequences between pre-treatment and on-treatment samples from a patient can partly overcome this limitation. Nevertheless, phenotypical confirmation assays[43;44] that can be performed without the need for a viral isolate will be a useful addition to the diagnostics of antiviral resistance of VZV.

Treatment failure and antiviral resistance

The study in chapter four aimed to investigate the occurrence and significance of persistence and antiviral resistance systematically by analyzing all episodes of VZV in hematological patients between 2007 and 2010. VZV episodes with a duration of at least 7 days were demonstrated in 59% of the episodes and were associated with complications in 50% of the episodes. Persistence was accompanied by antiviral resistance in 27% of the cases and some cases of resistant VZV concerned very complicated cases with unfavorable outcome.

Due to the retrospective and biased nature of the study, the significance of virological persistence without clinical persistence is unclear. However, it was shown that combined clinical and virological persistence may predict complications. Therefore, routine follow up after a VZV episode by PCR on blood samples has no proven additional predictive value, also because the consequence of asymptomatic persistence for antiviral treatment is unclear. Since antiviral resistance explained a relevant part of the persistent episodes, antiviral susceptibility testing should be performed timely and comprehensively (i.e. studying all affected body sites), to optimize patient management.

CYTOMEGALOVIRUS

Predictors of infection

Immunological determinants of the occurrence and outcome of CMV infection in transplant recipients have been studied widely.[45-67] Improved prediction on the basis of these immunological determinants of patients at risk for (complicated) CMV infections after transplantation could individualize prevention strategies.

As described in chapter eight, orthotopic liver transplant recipients were shown to have significantly increased rates of CMV infection if single-nucleotide polymorphisms (SNPs) were present in the gene for mannose-binding lectin 2 (MBL2) in the donor liver that are associated with decreased synthesis of MBL2. Furthermore, the risk of CMV infection was decreased in the presence of the minor allele of the Ficolin-2 (FCN2) gene in the donor liver that is associated with improved ligand binding. The joint genetic effect of these MBL2 and FCN2 genotypes in the donor liver was even stronger.

Interestingly, especially patients with the favorable genotype combination who received a liver with the unfavorable genotype combination had an increased risk for developing CMV reactivation compared to all other recipient-donor combinations. In contrast, recipients with an unfavorable genotype were not at increased risk of CMV. This suggests that some adaptation to or compensation for the potentially unfavorable genotypes of MBL2 and FCN2 occurs normally that is not transferred with the donor liver. The absence of this compensation in recipients with a favorable genotype combination, increases the risk of infection when receiving a liver from a donor with the unfavorable genotype combination.

The effects of MBL2 and FCN2 SNPs were most clear in CMV seropositive recipients of a liver from a CMV seronegative donor, who constitute a relevant proportion of liver transplant recipients. For this group, the optimal CMV prevention strategy has not been

defined and identifying immunological correlates of protection against severe CMV may aid in choosing the optimal strategy. However, as shown in our study the effect of SNPs in innate immunity genes is complex and their predictive potential should be studied in clinical trials before their use can be implemented in routine clinical practice.

Treatment

The optimal prevention strategy for CMV disease in renal transplant recipients has not been established yet.[68-71] In chapter seven the two most frequently applied regimens for the prevention of CMV disease in D+R- renal transplant recipients were systematically compared retrospectively. Patients treated in a purely preemptive strategy were compared with patients who were treated initially with three months of valganciclovir (vGCV) prophylaxis followed by a preemptive regimen. Prophylaxis effectively postponed CMV infections and reduced the percentage of patients reaching high CMV loads, as well as the AUC of CMV DNAemia, the duration of subsequent preemptive treatment episodes and the occurrence of treatment failure. No CMV end-organ disease occurred in either cohort.

The relatively mild course of CMV DNAemia during a preemptive regimen after initial prophylaxis in our study and in studies by others[72] demonstrated the effectiveness of regular CMV monitoring to prevent CMV disease. The severe outcomes of late-onset CMV disease reported in previous studies in which prophylaxis was not combined with a subsequent preemptive regimen emphasize the importance of regular monitoring after the end of prophylaxis in preventing late-onset CMV disease.[72;73] The optimal frequency and duration for monitoring remain to be studied.[74] Reluctance to include CMV monitoring is probably explained by the presumed costs of continued CMV monitoring after transplantation.[68-71] However, timely and thus more effective CMV treatment reduces the number of expensive hospital days for intravenous antiviral treatment and reduces long-term morbidity, including graft loss.[75-78]

Antiviral resistance

In case of treatment failure a rapid diagnosis of resistance is valuable. GCV resistance associated mutations in clinical isolates mainly map to the viral kinase gene UL97.[79-83] Sequence analysis is the fastest method for susceptibility testing of CMV. Novel techniques such as mass-spectrometry based comparative sequence analysis (MSCSA) combine the possibility of detection of all nucleotide variations within a target gene with reduced hands on time due to the automation of post-PCR processing and analysis.[84-86]

In chapter five, we investigated the applicability of an MSCSA method for automated high-throughput DNA sequence analysis for the detection of mutations in the UL97 gene. MSCSA was found to be equally accurate compared to conventional sequencing techniques and the sensitivity of mutation detection in a mixture was comparable as well. The accuracy of SNP detection by MSCSA was largely dependent on the quality (and quantity) of the sequences in the reference database, as performance improved considerably when the databases were supplemented with new sequences. Since MSCSA did not improve mutation (mixture) detection, its benefit lies mainly in its suitability for high-throughput analysis. With the relatively rare occurrence of GCV resistant CMV there is no such requirement. However, its ability to accurately detect resistance associated mutations in CMV is a proof of principle for its applicability in settings where larger amounts of samples can be expected, such as detection of resistance mutations in human immunodeficiency virus or influenza.

Currently, CMV resistance testing can be rapidly and easily performed by routine sequencing techniques. As shown in chapter six and seven, phenotypical confirmation of mutations is seldom required, since the number of mutations is rather limited and knowledge on the significance of these mutations is sufficiently available.

Treatment failure and antiviral resistance

The occurrence and possible causes of persistent CMV infection despite antiviral treatment were studied in two different cohorts of transplant recipients in chapter six and seven. In both studies, treatment failure was defined as the presence of at least 1000 copies/ ml of CMV DNA in plasma after a standard course of two weeks of treatment of CMV infection. Because the half-life of viral DNA in blood is probably very short,[27-31] plasma DNAemia is a correlate for recent viral replication.[87] This view is supported by the fact that viral loads decreased rapidly, with or without antiviral treatment, in many patients in our studies.

In D+R- renal transplant recipients treatment failure occurred in 52% of the treated patients (chapter seven). Undergoing a preemptive treatment regimen (i.e. without prior prophylaxis) and a high peak CMV load were found to be associated with treatment failure. In recipients of an allogeneic T-cell depleted HSCT who were at risk for CMV (donor and/or recipient CMV seropositive) treatment failure occurred in 55% of the treated patients (chapter six). The risk of treatment failure was increased during first treatment episodes and during the use of immunosuppressive medication. A high CMV load at the start of treatment was a predictor in univariable analysis only.

In both patient groups a comparable incidence of treatment failure of approximately 50% was found. This number is in accordance with previous studies in renal transplant recipients in which preemptive treatment resulted in a median time to clear DNAemia of 14-15 days and between 13 to 20 days, respectively.[88;89] For HSCT recipients varying rates of treatment failure have been reported from 7% up to 45%.[90-93] Different definitions of failure as well as differences in antiviral treatment regimens probably play a role in these variations. The similarity in the rates of treatment failure in the two very different patient groups studied in this thesis at least demonstrates that CMV replication often persist during antiviral treatment in transplant recipients.[69-71]

Predictors of treatment failure were different between the two types of transplant recipients. Apart from differences of patient characteristics and types of immune deficiencies between the two groups, variations in the analysis may account for some of the differences. In chapter seven, predictors were analyzed on a patient level, whereas in chapter six predictors were studied for each CMV episode. The latter method takes into account the effects of repeated measures per patients and increases statistical power and, in retrospect, would have been the preferred method in the study described in chapter seven. Nevertheless, as expected,[89;94;95] the height of the viral load at the beginning of antiviral treatment was a predictor (albeit univariable) in both studies. The relation with immunosuppressive medication and first episodes in HSCT recipients is logical, since lack of antiviral immunity allows for higher levels of viral replication hence increasing the time to clear CMV DNAemia.

The contribution of antiviral resistance to persistence was studied in both cohorts as well. Resistance was found in only one of 47 HSCT patients (2%) with CMV treatment. In D+R- renal transplant recipients resistance was found in four of 42 patients (9.5%) with CMV treatment. Hence, in both patient groups low although different rates of GCV resistance were found and four of five CMV infections with resistant virus were eventually cleared without switching antiviral therapy.

This implicated that in the majority of the cases persistent infection despite treatment is not due to antiviral resistance and that switching antiviral treatment to more toxic second line agents was often not necessary. Also, it demonstrated that antiviral resistance does not appear to be a negative consequence of a sequential prophylaxis-preemptive treatment regimen.[96] Our findings are in accordance with previous studies systematically addressing resistance,[91;92;96-98] but in contrast to studies on symptomatic CMV, in

which resistant CMV was found more frequently and more often caused CMV disease with an unfavorable outcome.[99;100] Explanations for this discrepancy may lie in the fact that in our studies resistance was studied regardless of symptoms and that CMV disease was effectively prevented in our patients. This emphasizes, again, the need for CMV monitoring to prevent disease. The results from both studies show that the role of antiviral resistance testing lies merely in reassuring clinicians to continue first line treatment awaiting viral clearance by the immune system. In addition, cessation of treatment in some cases of persistent infection may be safe as well and should be further studied.

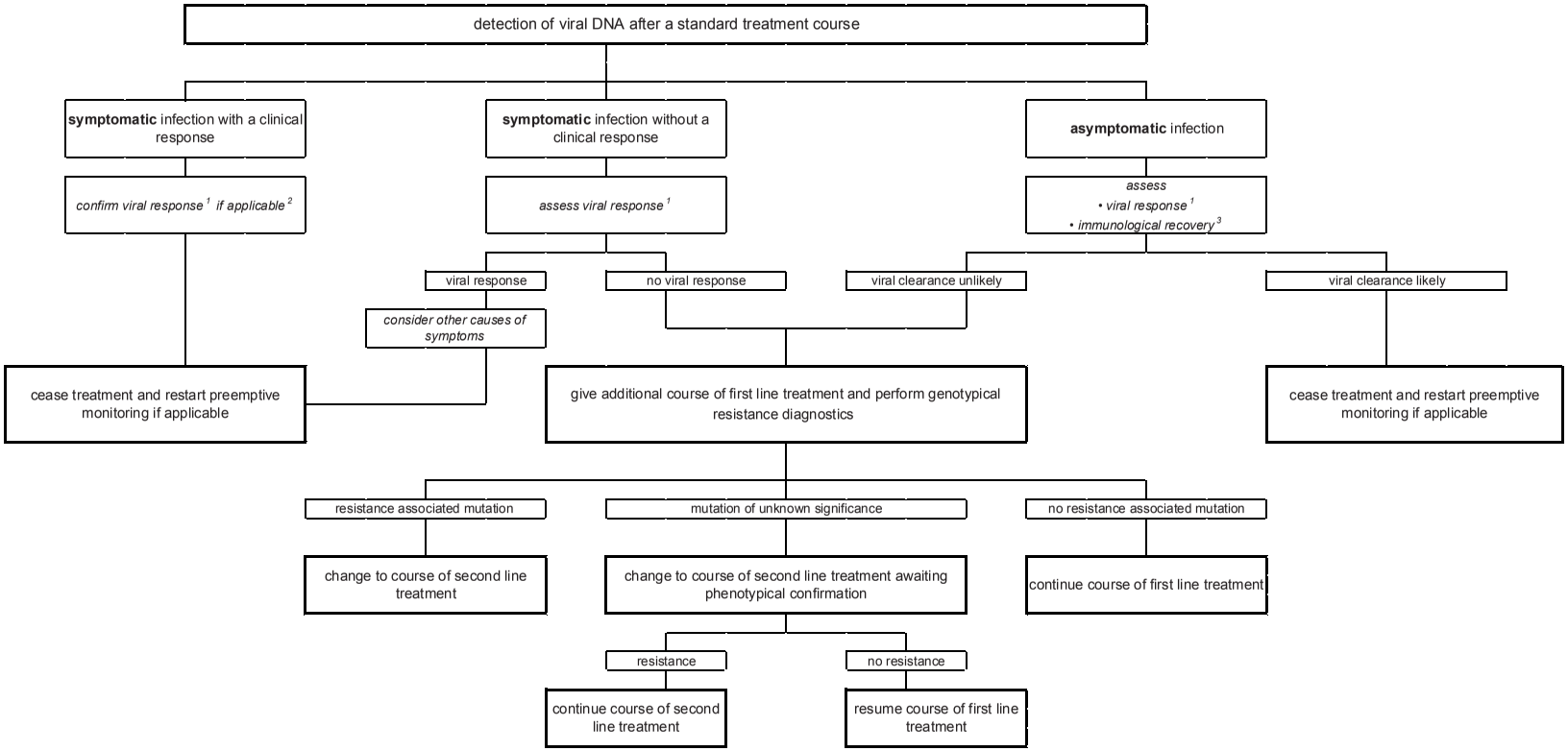
FUTURE DIRECTIONS

The aim of this research was to develop and improve diagnostic tools in order to rapidly diagnose HSV-1, VZV and CMV resistance to antiviral agents. Subsequently, the contribution of antiviral resistance and other predictors to persistent infections with HSV-1, VZV and CMV were studied.

Treatment failure

As shown in various chapters, persistence of herpesvirus DNA after a standard course of treatment is detected in about 50% of immunocompromised patients. If persistence is accompanied by clinical disease it is evident that additional antiviral treatment is required. However, the treatment of virological persistence without signs or symptoms of infections is a matter of debate. Of course, one should treat the patient and not the laboratory results, but in a preemptive setting this distinction is absent by definition. In addition, prolonged treatment of ongoing viral replication harbors the risk of selecting resistant virus. This emphasizes the limitations of our knowledge of the required treatment duration for herpesvirus infections after transplantation. In our studies the treating physicians usually have chosen to prolong treatment in patients with treatment failure. However, the duration of treatment of herpesvirus infections after transplantation is not based on controlled clinical trials and it is unknown whether treatment should be continued if viral replication persists after a course of treatment. Possibly, immune monitoring and viral dynamics may aid not only in deciding in whom to start antiviral treatment, but also in whom to safely end treatment.[94;101] At least one clinical trial is currently being performed on this topic.[101] Such studies may tailor the duration of antiviral treatment. For a flow chart on the suggested treatment of persistent herpesvirus infections, see Figure 1.

Figure 1. Flow chart for diagnostics and treatment in case of failure of antiviral therapy of DNA virus infections in immunocompromised patients.



Global approach to diagnostics and treatment in case of persistent DNA virus infections in immunosuppressed patients. [1]Assessment of viral response may include the viral load after treatment and the decrease in viral load in response to the given treatment. [2]In case of severe symptomatic disease. [3]Assessment of immunological recovery may include total and virus-specific T-cell counts and the possibility to decrease the use of immunosuppressive medication. Details may differ per virus.

It appears from the presented observations that antiviral immunity is the main determinant of treatment response. Interesting and promising in this regard are the current trials (http://clinicaltrials.gov/) on the effectiveness of pre-transplantation CMV and VZV vaccination strategies in preventing the occurrence or changing the course of these herpesvirus infections after transplantation.[102]

Antiviral resistance

Also shown in various chapters is the fact that persistent herpesvirus infection is explained by resistance in only a minority of the cases. Often, resistance was associated with a severe clinical course of the infection, but sometimes resistant viral isolates cleared spontaneously without appreciable clinical problems. These findings emphasize the need for rapid and adequate diagnostics of antiviral resistance in cases of persistent clinical and virological infection. Susceptibility testing will rule out resistance in most cases thus avoiding the need for second line agents with their associated toxicity and need for intravenous administration. In addition, it will optimize treatment in patients with resistant virus who not seldomly have serious organ manifestations.

Diagnosing antiviral resistance was found to be most straightforward in CMV where simple sequencing analysis suffices in most instances. Web based software tools, such as ReCall (RECall beta v2.6, http://pssm.cfenet.ubc.ca/home/index) can be applied for herpesvirus sequence analysis and may facilitate sequence analysis in routine diagnostics. For HSV and VZV, a two step approach is required, starting with sequence analysis and followed by phenotypical confirmation if mutations of unclear significance are found. An additional difficulty for VZV is the fact that usually viral isolates cannot be obtained. The latter point deserves further assay development. Possible compartmentalization demands investigation of virus in all affected body sites. For a flow chart on suggested diagnostics in case of persistent herpesvirus infections, see Figure 1.

There is a relevant need for less toxic and oral alternatives for antiviral treatment in case of antiviral resistance. For HSV several trials exploring novel antiviral drugs have been done (e.g. helicase-primase inhibitors) and are ongoing (e.g. NB-001, BTL TML HSV, http://clinicaltrials.gov/) and pre-clinical studies have been performed for other new drugs.[103;104] For CMV novel agents for treatment including CMX001 are being investigated.[105-107] For VZV an as yet unpublished clinical trial on the effectiveness of FV-100 has been performed (http://clinicaltrials.gov/). This increases the number of options for antiviral treatment in case of resistance. As a consequence, susceptibility testing needs to be adapted continuously to include novel antivirals.

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