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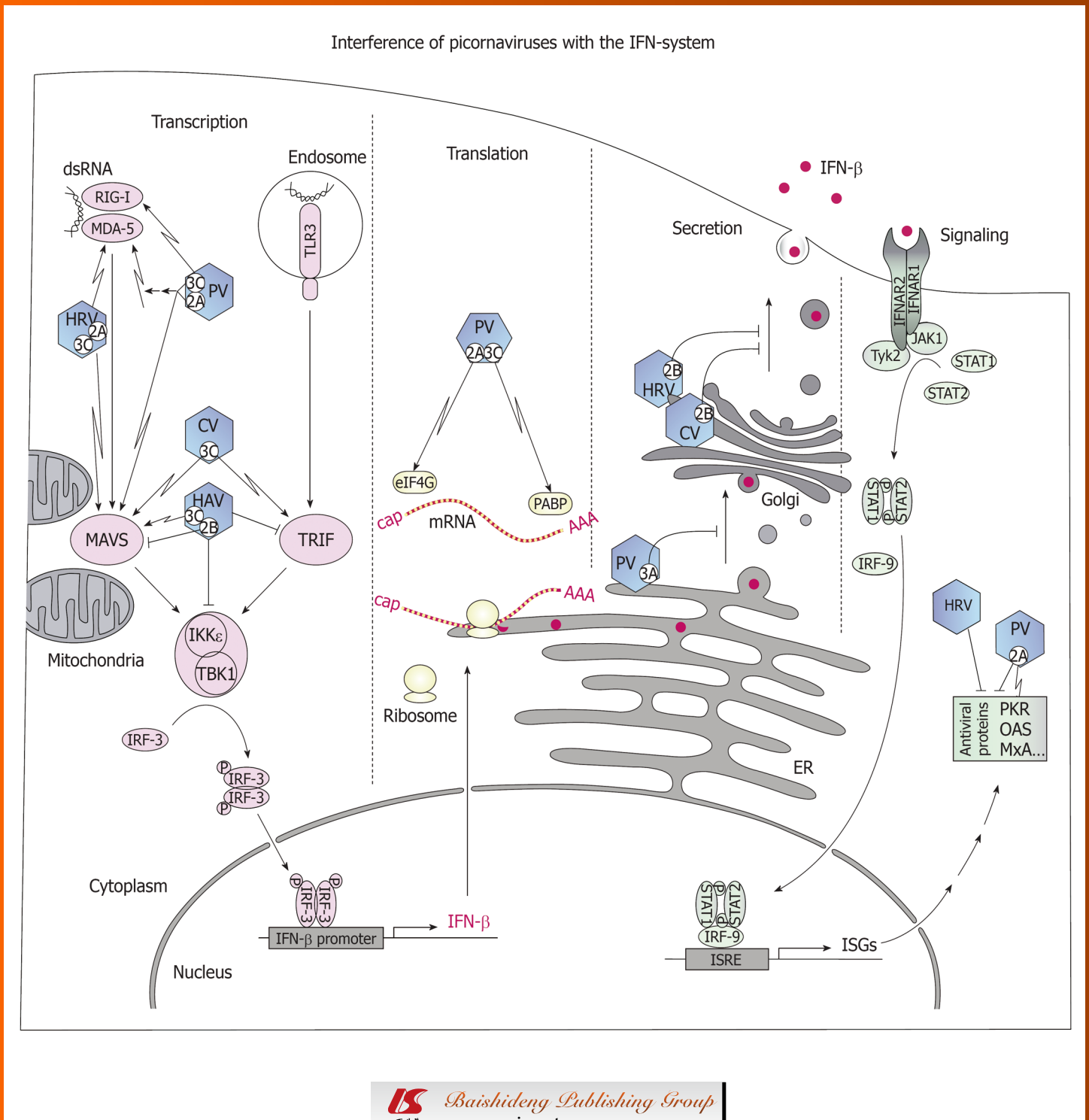

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EDITORIAL

Molecular and clinical aspects of hepatitis D virus infections

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Abstract

Hepatitis D virus (HDV) is a defective virus with circular, single-stranded genomic RNA which needs hepatitis B virus (HBV) as a helper virus for virion assembly and infectivity. HDV virions are composed of a circular shape HDV RNA and two types of viral proteins, small and large HDAgs, surrounded by HBV surface antigen (HBsAg). The RNA polymerase Ⅱ from infected hepatocytes is responsible for synthesizing RNAs with positive and negative polarities for HDV, as the virus does not code any enzyme to replicate its genome. HDV occurs as co-infection or super-infection in up to 5% of HBsAg carriers. A recent multi-center study highlighted that pegylated interferon α-2a (PEG-IFN) is currently the only treatment option for delta hepatitis. Nucleotide/nucleoside analogues, which are effective against HBV, have no relevant effects on HDV. However, additional clinical trials combining PEG-IFN and tenofovir are currently ongoing. The molecular interactions between HDV and HBV are incompletely understood. Despite fluctuating patterns of HBV viral load in the presence of HDV in patients, several observations indicate that HDV has suppressive effects on HBV replication, and even in triple infections with HDV, HBV and HCV, replication of both concomitant viruses can be reduced. Additional molecular virology studies are warranted to clarify how HDV interacts with the helper virus and which key cellular pathways are used by both viruses. Further clinical trials are underway to optimize treatment strategies for delta hepatitis.

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Key words: Hepatitis D virus; Hepatitis B virus; Delta hepatitis; Liver cirrhosis; Hepatocellular carcinoma; Hepatitis B virus surface antigen

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INTRODUCTION

The hepatitis D virus (HDV) causes chronic or fulminant hepatitis in patients, as a co- or super-infection in hepatitis B virus (HBV) infected individuals[1,2]. Experimentally, the "delta virus" can also infect chimpanzees or woodchucks who have already been infected with HBV or woodchuck hepatitis virus (WHV), respectively[3]. HBV/HDV co-infection is a clinically very deleterious condition as it commonly leads to progression of hepatic fibrosis, cirrhosis and increase the risk of hepatocellular carcinoma[4]. The mechanism by which HDV promotes hepatic malignancies are unclear, however it has been shown that the virus has negative effects on hepatocytes' growth and viability[5].

HDV, with a spherical shape and a virion size of 36 nm, is a satellite virus with a circular RNA of negative polarity which requires obligatory a helper function to propagate[1,2]. This helper function is usually provided through HBV by sharing its envelope proteins[2]. Some *in-vivo* studies have shown that other *Orthohepadnaviri-*

dae members like WHV can also play the same role as HBV[6,7]. There are some arguments that interactions between HDV and its helper virus might not be limited to surface protein supplies, but also other unknown mechanisms[2]. With respect to the size of the genome (1679 bp RNA), the rolling circle mechanism of replication and its high GC nucleotide content, HDV is very unique among animal viruses and looks very similar to viroids (a family of helper-independent plant pathogens)[8].

VIRAL STRUCTURE

The HDV ribonucleoprotein (RNP) is surrounded by a mixture of host cell-derived lipids and HBV surface proteins (HBsAg)[9]. HDV has a small single stranded RNA genome of 1679 nucleotids[10]. Due to the high degree of intramolecular base pairing in the HDV genome, the RNA folds to an un-branched, rod like structure[11]. HDV's circular RNA forms a complex with two viral-encoded proteins, small (195 amino acids) and large (214 amino acids) HDV-antigens (HDAg-S or p24, and HDAg-L or p27, respectively)[6,9,11]. Delta proteins have identical amino acids except for 19 additional residues at the C-terminus of HDAg-L[9].

The small delta protein acts as a trans-activator for initiation of genomic RNA replication[12]. It also undergoes post translational modifications like methylation, acetylation and phosphorylation to mediate viral mRNA transcription in its modified form[10,13]. HDAg-L is a late protein which inhibits viral RNA replication and transcription of viral proteins, interacts with HBV surface antigens and accelerates the assembly of new virions[6,9,11]. The capacity of binding to HBsAg is not only limited to HDAg-L protein but also occurs with HDAg-S. In fact, in the presence of HDAg-S the packaging level of HDV raises up to 3 to 4 fold[9]. Moreover, there is a conserved molar ratio of delta antigen to HDV genomic RNA in infected liver tissues as well as in HDV particles[10].

MOLECULAR INTERACTIONS BETWEEN HBV AND HDV

HBV, with a 3.2 kb partial double stranded DNA, plays the role of a HDV helper virus in HBV/HDV-infected hepatocytes. From the four overlapping reading frames in the HBV genome one encodes viral surface envelope proteins[14]. This region contains (from amino- to carboxyl-end) the pre-S1 (119 aa), pre-S2 (55 aa) and S (226 aa) domains[9]. These domains encode the large (from pre-S1, pre-S2 and S), middle (from pre-S2 and S) and small (from S) HBV surface proteins which are all in the same translational frame with different start codons (N-terminals)[6,9].

Although the S-HBsAg alone is sufficient for virion development due to its self-assembling trait (which most of the time leads to empty envelope particle formation), the presence of large HBsAg is necessary for both HBV and HDV to infect other cells[6,9]. *In-vivo* studies have shown that all three HBsAg proteins are present in HDV particles[9]. Delta proteins can bind to the S domain of HBV envelope as well as the L4 region which is located in the pre-S1 domain of HBsAg (amino acid residues 86-108). This is the same region which HBV core proteins interact with[9].

The surface protein coding region of HBV overlaps with polymerase encoding genes of HBV, so any mutation in this region may affect both polymerase and HBsAg activities[6]. In fact, mutations in the HBV polymerase gene that also affect correspondingly the HBV surface antigen coding region, may therefore have effects on HBV as well as on HDV replication efficacy. Not all consequences of HBV mutations for HBV/HDV viral replication are currently understood, but limited data exist for some of the most common clinical variants (Table 1). Effects of mutations within the HBV polymerase and surface proteins on HDV secretion. LMV stands for Lamivudine. Data are mainly derived from molecular interaction studies *in vitro*[6,15,16]. While the lamivudine-resistant mutant rtM204V improves HDV secretion, another resistance-conferring mutant like rtM204I (corresponds to sW196L/S/stop) diminishes HDV production[6], which is due to the importance of the codon 196 in the HBV surface antigen for HDV packaging[6,15].

Mutations in C-terminal region of the envelope proteins (especially between amino acid residues 163 to 224) can severely reduce HDV assembly[16]. HBsAg mutations, selected by antiviral agents, are also located in this region[6]. These mutations can affect the level of HDV virion secretion out of the cells in *in-vitro* experiments[6,16].

Although it is known that HBV/HDV-coinfected patients have an unfavorable clinical outcome, the exact role of HBV and HDV in liver disease progression has been controversially discussed, because contrasting results have been published about the role of each one of the viruses[10,12]. Some authors suggested that the underlying HBV infection is aggravated by concomitant presence of HDV in hepatocytes with synergistic deleterious effects on cell survival[12,17], while others indicated that HDV, which often suppresses HBV, is most critical for liver disease and malignant transformation[5,10,12]. From a molecular point of view, it is very likely that both viruses do not replicate independent from one another, but modulate each other's replication level and also pathogenicity[2]. Suppressed HBV replication, but also high or fluctuating loads of both viruses in the serum of co-infected individuals have been reported[2,12]. A recent longitudinal study investigating quantitatively HBV and HDV viral loads in co-infected patients showed that in spite of cross-sectional studies, in which there is a significant positive association between HBsAg level and HBV-DNA or HDV-RNA, HBsAg has longitudinally fluctuating levels in the presence of HDV[12]. Apparently, there is not always an inhibitory effect of HDV on HBV replication as sometimes HBV shows significant inhibitory actions on HDV in HBV/HDV double infection as well.

Table 1 Molecular interactions between hepatitis B virus and hepatitis D virus

HBV polymerase mutation(s)	Corresponding HBsAg mutation(s)	Clinical relevance	Effect(s) on HDV
rtM204V	sI195M(A)	LMV resistance	Enhanced HDV secretion
rtM204I	sW196L/S/stop	LMV resistance	S, L: no HDV secretion
rtD205H	sW196F	Selected during LMV treatment with reduced binding to anti-HBsAg antibodies	Reduced HDV secretion
rtV173L	sE164D	Selected during LMV treatment with reduced binding to anti-HBsAg antibodies	Reduced HDV secretion
rtV173L/rtM204V	sE164D/sI195M	Selected during LMV treatment with reduced binding to anti-HBsAg antibodies	Support HDV secretion

LMV: Lamivudine; HDV: hepatitis D virus; HBV hepatitis B virus; HBsAg: HBV surface antigen.

This observation might be due to some changes in HBV surface antigen region interacting with HDV[12]. Overall, the molecular mechanisms underlying HBV/HDV interactions leading to the progression of the disease are still uncertain and require intense further investigations.

PROTEIN MODIFICATIONS OF HDV

The level of HDV replication and pathogenicity is not only influenced by interactions with HBV and HBV mutants, but might be also impacted by modifications of the HDV proteins as well. As such, some amino acid residues in S- and L-HDAg appear to be critical for posttranslational modifications[1,10]. Of these residues Arg-13, Lys-72 and Ser-177 in S-HDAg undergo methylation[18], acetylation[19] and phosphorylation[20], respectively[1,10].

There are also multiple lysin residues throughout the whole S-HDAg as well as 66 amino acids at the N-terminal part of this protein which act as sumoylation sites of the protein. Sumoylation is a reversible process which has implications for cell cycle progression, nuclear import, regulation of transcription, protein turnover and other cell biology functions. In case of HDV, sumoylation enhances G-RNA and mRNA synthesis by unknown mechanism but has no effect on antigenomic RNA (AG-RNA) synthesis[1].

In L-HDAg Cys-211 gets isoprenylated for virus assembly[1,21,22]. Deletion of 15 amino acids upstream of the isoprenylation site would also lead to the eradication of viral replication[21]. Moreover, some mutants of HDV have been observed which can only replicate in the presence of wild-type HDV, called "defective" viruses[23].

HDV REPLICATION

The replication cycle of HDV is schematically summarized in Figure 1. N-terminal residues of Large Hepatitis B surface antigen mediate the entry of HDV into the hepatocytes[24]. Once delta virus enters the cell it gets uncoated, and the accompanying S-HDAg leads the HDV nucleoprotein complex to the cell's nucleous[10,11]. HDV has a mechanism of double rolling circle amplification. For this, the virus needs an RNA-dependent RNA polymerase activity which in the majority of RNA viruses, but not in the case of HDV, is carried out by virally encoded enzymes[25]. Exceptionally, the delta hepatitis virus is capable of using host RNA polymerases to amplify its genome[25].

The HDV genomic strand undergoes RNA-dependent RNA synthesis, more likely by nucleolar RNA-POL-I[1,11], to produce multimeric full-length intermediate RNAs or AG-RNAs. These molecules then serve as templates for cellular RNA Pol-Ⅱ to generate HDV genomic RNA again through another rolling circle step[11]. Both genomic and anti-genomic strands of HDV contain 85 nucleotides with ribozyme activity which enables the virus to self-cleavage and to ligate its circular RNA[2]. There is also the possibility of producing small segments of RNA transcripts, from both genomic and anti-genomic HDV RNAs which have been consumed to contribute to viral replication[25].

Genomic RNA is also transcribed into an mRNA (0.8 kb) which encodes the HDAgs[1]. This step distinguishes HDV from viroids since they do not produce any protein. This event has also been shown to take place in the nucleus, the same place at which G-RNA synthesis happens[1]. It means that different cellular machineries are mediating HDV genomic RNA/mRNA and HDV anti-genomic RNA synthesis, which are localized in the nucleus and nucleolus of the host cells, respectively[1,11,26,27]. Modified small HDAg intermediates viral mRNA transcription[10,13].

During small HDAg production, an RNA editing event happens at position 1012 by double-stranded RNA-specific adenosine deaminase[22], resulting in alteration of the stop codon of the HDAg-S open reading frame (ORF) and translation extension for additional 19 amino acids[11]. This edition is very essential for the virus since it creates an ORF for the large delta antigen to be translated[22]. The extra 19 amino acid sequence of p27 is poorly conserved among different HDV isolates. However a CXXX motif inside this region causes prenylation of the protein, facilitates protein-protein interactions and directs it to the host cell membrane[28]. Defective mutants of this motif are not able to interact with HBsAgs and to be packaged[28,29]. Expression of L-HDAg initiates interactions with HBV surface proteins and HDV RNP encapsidation[9,11].

TREATMENT OF DELTA HEPATITIS

Chronic hepatitis D, "delta hepatitis", principally demands effective therapy, due to the adverse natural history of chronic HDV infection with more severe liver disease, rapid progression to cirrhosis, increased hepatic decompensation and higher mortality rates compared to HBV

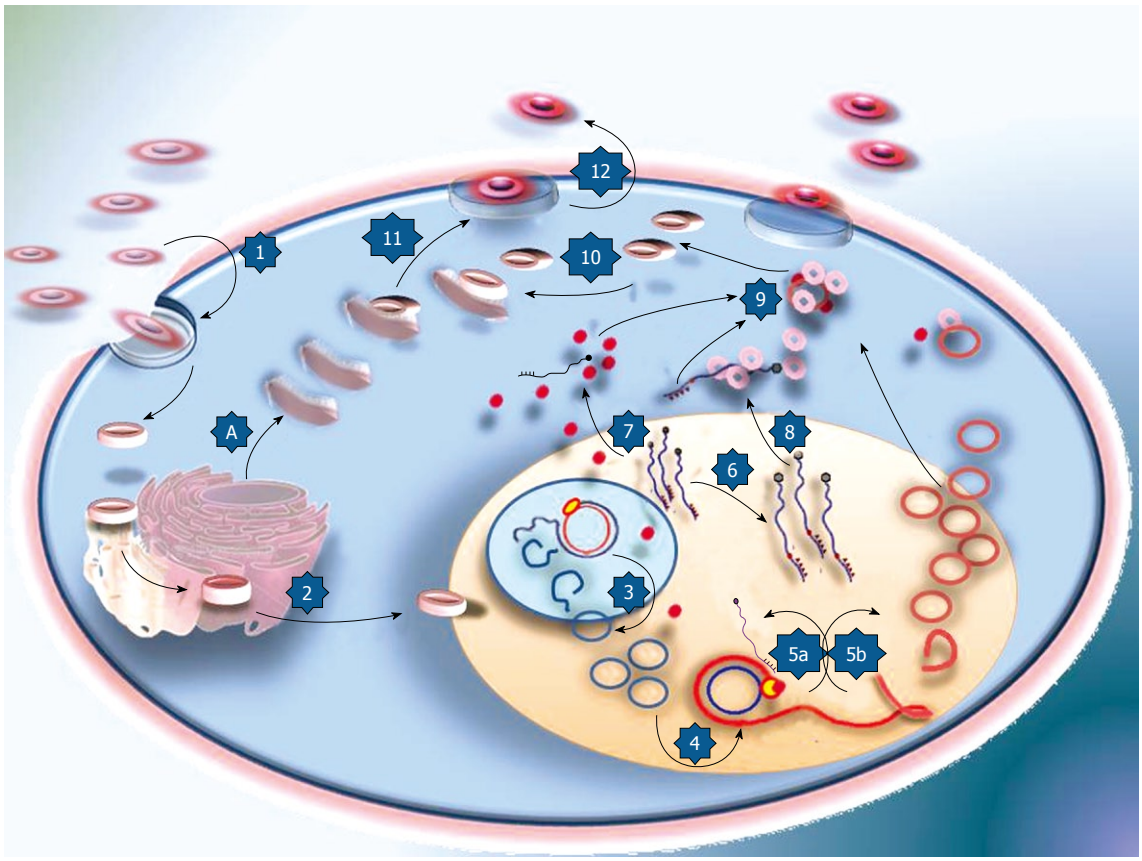

Figure 1 Hepatitis D virus life cycle in hepatocytes in the presence of hepatitis B virus. Schematic summary of the current concept of hepatitis D virus (HDV) replication cycle. The entry of HDV particles into hepatocytes is mediated by the attachment of hepatitis B virus (HBV) surface antigens coating HDV nucleoprotein to the host cell receptors, followed by endocytosis and uncoating of the virions (1). HDV nucleoprotein complex is leaded to the nucleus by accompanying S-HDAg, then the complex arrives to the nucleolus where RNA Pol Ⅰ exists (2). Multimeric full-length antigenomic RNAs (AG-RNAs) are transcribed from HDV genomic RNA, likely by RNA Pol Ⅰ. Circular AG-RNA molecules which are created by ribozyme activities of AG-RNA itself move to the nucleoplasm (3). AG-Strands serve as templates for RNA Pol Ⅱ (4). RNA Pol Ⅱ starts generating mRNA from genomic strands (5a) and full-length transcripts from AG-strands (5b). 6: Due to RNA editing at position 1012 of S-HDAg exerted by double-stranded RNA-specific adenosine deaminase, the open reading frame of these mRNA molecules extends for additional 19 amino acids, which lead to the production of Large delta antigen (6). mRNA molecules coding for small and large delta antigens move to the cytoplasm and are translated to relevant proteins (7,8). Small delta antigens activate genomic RNA replication (7), while large proteins promote virion assembly (8). Viral proteins form nucleoprotein complexes with HDV genomic RNAs (9). L-HDAg in HDV ribonucleoprotein complex interacts with existing HBV surface proteins in the cell (10). After HDV encapsidation by HBV surface proteins (11), complete virions leave the cell through exocytosis (12). HBV surface proteins bud through the endoplasmic reticulum or golgi body membranes of the host cell (A).

monoinfection[30,31]. However, the therapy of chronic hepatitis D infection is a major challenge, because there is no specific virus inhibitor. At the moment, there is only one approved therapy with IFN α or PEG-IFN α, respectively, available[30]. A lot of different substances have been investigated; Table 2 gives an overview of the results of selected major clinical trials.

The recent standard therapy for delta hepatitis is PEG-IFN, administered 180 μg s.c. once per week for a period of 48 wk. Traditionally, conventionally IFN proofed to be effective in chronic hepatitis D in the early 1990s[32-35]. Also, a placebo-controlled trial reported in 2005 could show a benefit for conventional IFN in a small cohort[36]. But nevertheless response rates in matters of sustained virological responses (SVRs) are low and therapy efficacy seems to be proportional to the dose and duration of treatment[37]. In a single case with a 12-year permanent therapy with 5 million units IFN daily a HDV RNA clearance could be achieved after several years accompanied with anti-HBs seroconversion. This was attended by improvement of liver histology (initially cirrhosis, after 10 years no abnormalities)[38]. Interestingly Yurdaydin *et al*[39] could not find a benefit for IFN treatment over 2 years instead of 1 year. However long-term data (12 years) showed a benefit of high-dose IFN α therapy (9 million units three times per week) with even regression of advanced hepatic fibrosis[40].

Due to the advantageous pharmacodynamics and pharmacokinetics, PEG-IFN clearly provided a benefit in HDV therapy. In 2006 three small studies could show effectiveness for this type of treatment[41-43], but nevertheless SVR rates remained low (17% to 43%) overall. In a multicentre randomised landmark trial published in 2011, Wedemeyer *et al*[44] achieved 28% SVR. These differences in SVR rates compared to prior smaller trials might be due to baseline clinical, demographical and virological characteristics[30]. Farci[37] proposed to divide patients into IFN responders and non-responders. Additionally,

Table 2 Selected clinical trials on delta hepatitis therapy

Ref.	Drug used	Dosage	No. of patients included	Main result (s)
Garripoli *et al*[45], 1994	Ribavirin monotherapy	15 mg/kg for 16 wk	9	Ribavirin did not show significant antiviral effects in chronic hepatitis D
Wolters *et al*[49], 2000	LAM + IFN add-on	LAM 100 mg at least for 24 wk; afterwards combination therapy with IFN 9 MU/d for 4 wk, followed by 9 MU 3 times/wk for 12 wk	8	Neither LAM alone nor the addition of IFN was capable of reducing HDV
Yurdaydin *et al*[47], 2002	Famciclovir	500 mg for 6 mo	15	Not effective
Farci *et al*[40], 2004	High-dose IFN α *vs* low dose IFN *vs* no treatment	High dose: 9 million units 3 times/wk, low dose 3 million units 3 times/wk for 48 wk	36	High-dose IFN α significantly improves long term clinical outcome and survival
Kaymakoglu *et al*[46], 2005	IFN α + ribavirin	IFN 10 MU 3 times/wk, Ribavirin 1000-1200 mg/d for 24 mo	19	Addition of Ribavirin to IFN-α does not increase response rate in patients with CHD
Niro *et al*[36], 2005	LAM *vs* placebo	100 mg LAM for 52 wk	31	HDV viraemia was unaffected, even in patients when HBV replication was lowered by LAM therapy
Erhardt *et al*[43], 2006	PEG-IFN	1.5 μg/kg PEG-IFN per wk for 48 wk	12	PEG-IFN is a promising treatment option in chronic hepatitis D
Castelnau *et al*[41], 2006	PEG-IFN	1.5 μg/kg PEG-IFN per wk for 12 mo	14	PEG-IFN is safe and efficient for HDV treatment
Niro *et al*[42], 2006	PEG-IFN mono *vs* combination therapy with ribavirin	1.5 μg/kg PEG-IFN per wk; 800 mg ribavirin; 48 wk mono or combination therapy, afterwards 24 wk PEG-IFN mono	38	Ribavirin had no effect
Yurdaydin *et al*[50], 2008	LAM *vs* LAM + IFN *vs* IFN mono	IFN 9MU 3 times/wk, LAM 100 mg; totally 12 mo therapy; for combination therapy 2 mo LAM mono, afterwards 10 mo combination	39	Addition of LAM to IFN is of no additional value; both (IFN mono/IFN + LAM) are superior to LAM mono
Mansour *et al*[52], 2010	PEG-IFN, add-on tenofovir und emtricitabine after 2 mo	PEG-IFN 180 μg/wk; tenofovir 300 mg/d for 10 mo	1	Combination therapy with PEG-IFN and nucleoside/tide analogue seems to be more effective than IFN alone
Wedemeyer *et al*[44], 2011	PEG-IFN mono *vs* adefovir *vs* combination PEG-IFN + adefovir	PEG-IFN 180 μg/wk; adefovir 10 mg/d for 48 wk	90	PEG-IFN α-2a with or without adefovir resulted in sustained HDV clearance in about 25%

LMV: Lamivudine; LAM: Lamivudine; CHD: Coronary heart disease; HDV: hepatitis D virus; HBV hepatitis B virus; PEG-IFN: Pegylated interferon.

IFN responders might be distinguished in early- and late-responders. The latter could possibly benefit from a prolonged treatment, because HDV decrease might occur late, even after the end of treatment[44]. Clear predictors of response to IFN have not been identified, but viral load determination at 6 mo of treatment might be helpful[41,43]. Even in virological non-responders, Erhardt *et al*[43] observed a stabilization of histological liver score under therapy so that IFN might be beneficial.

Because of the poor results and high rates of adverse events like flu-like symptoms or bone marrow suppression with anemia or neutropenia, dose reduction or discontinuation of treatment are common upon PEG-IFN therapy, corroborating the urgent need for therapeutic alternatives.

Major problems in developing new treatment strategies are that there are no specific therapeutic targets like a virus polymerase and that potentially two viruses have to be treated at the same time[30]. A lot of different approaches have been investigated over the last years. Ribavirin as a monotherapy or in combination with IFN could not show a benefit[42,45,46], likewise Famciclovir[47] and acyclovir[48] had no effect. Different studies could not show an advantage of lamivudine as a monotherapy or combination therapy[36,49,50]. Because of a significant decrease in cccDNA levels accompanied with a reduction in serum HBsAg titers in long-term adefovir therapy[51], the well-known HBV-effective nucleotide analogue adefovir dipivoxil was thought to be a potential anti-HDV drug as well. Interestingly, in the recent HIDIT-1 study Wedemeyer *et al*[44] could not find superiority compared to PEG-IFN monotherapy. Thus, nucleoside/nucleotide analogue treatment is not recommended at the moment in patients with suppressed or low HBV replication. In a single case report, a patient achieved a SVR accompanied with anti-HBs seroconversion after add-on therapy of tenofovir and emtricitabine to PEG-IFN[52]. Therefore, further studies are required to investigate the role of nucleoside/nucleotide analogues with high resistance barrier like entecavir or tenofovir. The HIDIT-2 trial, for instance, combines PEG-IFN with tenofovir for the treatment of HDV, and results from this trial are anticipated within the next years.

Because of different patterns in replication of HDV and HBV (active HDV/inactive HBV 70%, active HDV and HBV 23%, inactive HDV/active HBV 4%, both inactive 3%)[53] which vary over time a close treatment surveillance and an individually adopted therapy is likely to be essential. Possibly, patients with a high HBV replication might benefit from a therapy with nucleoside/nucleotide

analogues, because of the long-term HBsAg reduction observed with these agents. Sheldon *et al*[54] could show that in a long-term study (median 6.1 years) of HIV/HDV/HBV co-infected patients who where treated with anti-HBV drugs 13 of 16 patients had reduced HDV viremia and ALT levels. Even three of these achieved undetectable HDV RNA and normal ALT levels. This is especially important considering that high levels of HBV-DNA in HDV/HBV co-infected patients lead to more severe liver damage than those with low viremia[55].

Another promising approach is the use of prenylation inhibitors. Bordier *et al*[56] used a farnesyltransferase inhibitor (FTI) because prenylation of the large delta antigen - especially the prenyl lipid farnesyl, which was found on the delta antigen[57] - seems to play an essential role for the virus assembly and release. In this study, a complete clearance of HDV viremia with FTI was achieved[56]. Orally taken FTIs have been developed with a relative lack of toxicity in human phase I / II studies[58] and might be a potential new substance group for treatment of chronic HDV infection.

In a pilot study of chronically infected woodchucks clevudine was capable to reduce WHV cccDNA with reduction in WHsAg. Moreover, they could achieve in 75% of HDV infected woodchucks undetectable HDV RNA with clevudine treatment[59]. An HBsAg titer reduction by reducing the cccDNA during clevudine therapy was also found in humans[60]. In comparison to lamivudine, clevudine seems to be superior in HBeAg positive HBV. Compared to entecavir, in chronic HBV infected patients clevudine could reduce viral load similar than entecavir, but higher rates of virological breakthrough and significantly more myopathy was observed[61], indicating that clevudine has a higher adverse event profile.

Potential novel strategies for an anti-HDV treatment might be an HDV receptor blockade, which is thought to be the same receptor like HBV. Also, a modulation of the balance between S-HDAg and L-HDAg and especially modification of post-translational changes of HDAg, which effects the viral life cycle, might be a promising target. Another approach is the reduction of HBsAg, which might be associated with clearance of HDV RNA. Vietheer *et al*[6] for instance could show that mutations in the HBsAg lead to an inhibition of HDV particle secretion. But it should be taken into consideration that HDV, once it got into the cell, can replicate without HBV[62-64], so that an HBsAg reduction might inhibit new infections of cells, but can theoretically not by itself promote the clearance from already infected cells. Also other IFN types, like IFN λ[30], should be evaluated for their efficacy in HDV.

At the moment, the current standard therapy for chronic HDV infection is PEG-IFN α-2a 180 μg s.c. weekly. Wedemeyer *et al*[44] proposed a treatment for 48 wk. If there is a high viremia with positive anti-HDV IgM after this treatment period, a response seems to be unlikely. If there is a reduced viremia, decreased IgM antibody titers or transaminases patients might benefit from extended therapy for 72 wk. Patients with a high HBV replication might benefit by nucleoside/nucleotide therapy. Also a spontaneously HDV clearance might appear with spontaneously seroconversion to anti-HBs (0.25% annual rate). Nevertheless, therapy is needed in delta hepatitis, because HDV replication is an independent predictor of mortality[65] and lack of treatment is a predictor of an unfavourable outcome[53]. Even 8/35 patients with an SVR developed a HCC in long-term[65], especially elderly are at a specific risk[66].

The aim of therapy is a HDV RNA clearance, seroconversion to anti-HBs and avoiding imminent complications like cirrhosis. Once a stage of liver cirrhosis has developed, the viral clearance will have limited influence on the further course of liver disease. Half of patients who develop cirrhosis later will progress to liver failure[53]. To cure patients, long-term IFN treatment is required for undetectable HDV RNA and further treatment is required for HBsAg loss[67]. During therapy, monitoring of HDV RNA and HBsAg might help in the surveillance of therapy, although HDV RNA does not correlate with activity or stage of liver disease[68].

Overall, more long-term data as well as a better understanding of the viral life cycle and HDV/HBV interactions are needed for an efficient HDV treatment. Until then, HDV infection obviation should be a major focus of health care measures by preventing delta hepatitis using vaccination against HBV, especially in countries with high HDV prevalence[31].

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GUIDELINES FOR BASIC SCIENCE

Selection of RNAi-based inhibitors for anti-HIV gene therapy

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Abstract

In the last decade, RNA interference (RNAi) advanced to one of the most widely applied techniques in the biomedical research field and several RNAi therapeutic clinical trials have been launched. We focus on RNAi-based inhibitors against the chronic infection with human immunodeficiency virus type 1 (HIV-1). A lentiviral gene therapy is proposed for HIV-infected patients that will protect and reconstitute the vital immune cell pool. The RNAi-based inhibitors that have been developed are short hairpin RNA molecules (shRNAs), of which multiple are needed to prevent viral escape. In ten distinct steps, we describe the selection process that started with 135 shRNA candidates, from the initial design criteria, *via* testing of the *in vitro* and *in vivo* antiviral activity and cytotoxicity to the final design of a combinatorial therapy with three shRNAs. These shRNAs satisfied all 10 selection criteria such as targeting conserved regions of the HIV-1 RNA genome, exhibiting robust inhibition of HIV-1 replication and having no impact on cell physiology. This combinatorial shRNA vector will soon move forward to the first clinical studies.

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Key words: Human immunodeficiency virus type 1; RNA interference; Gene therapy; "Human Immune System" mouse; Lentivirus

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INTRODUCTION

After discovery of the mechanism of RNA interference (RNAi) in C. elegans in 1998[1], several RNAi approaches have been developed for use in therapeutic strategies, e.g., against inherited diseases or infectious pathogens[2]. The cellular RNAi pathway leads to the processing of small noncoding microRNAs (miRNAs) that regulate cellular gene expression at the post-transcriptional level to control cell differentiation and development[3]. This pathway may be primed by artificial short hairpin RNAs (shRNAs) that are produced in the cell from a transgene and processed into small interfering RNAs (siRNAs)[4]. Ready-to-use siRNAs can also be synthesized chemically and transfected into cells. Perfect base-pairing of the designed siRNA with the specific mRNA target results in cleavage of the latter by the RNA-induced silencing complex (RISC)[5]. Topical delivery of siRNAs in the lungs might be feasible for the treatment of acute infections with e.g.,

influenza A virus or the respiratory syncytial virus. However, chronic infections caused by pathogens such as the human immunodeficiency virus type 1 (HIV-1), hepatitis B virus and hepatitis C virus will require the continuous expression of RNAi inhibitors from a therapeutic transgene.

HIV-1 is replicating in cells of the human immune system, resulting in a constant depletion of the CD4+ T cells that contributes to the eventual progression to AIDS. Anti-HIV gene therapy aims to protect this indispensable cell pool from virus infection and destruction, which should lead to a (partial) reconstitution of the immune system. Due to the chronic nature of HIV-1 infection, cells must be protected life-long against HIV-1, which can be achieved by a stable RNAi gene therapy against the HIV-1 RNA genome. Apart from RNAi approaches, other antiviral strategies can be utilized such as ribozymes, antisense RNAs, dominant negative protein variants, decoy RNAs, or combinations of RNAi, ribozymes and RNA decoys[6]. However, the simultaneous use of multiple RNAi inhibitors seems one of the most promising approaches for a potent and durable therapy[7]. The therapeutic protocol that we have in mind starts with the isolation of blood mobilized CD34+ hematopoietic progenitor cells from HIV-infected patients, followed by *ex vivo* transduction with an shRNA-expressing lentiviral vector that stably integrates in the host cell DNA, and re-injection of the modified cells into the patients. Target cells for HIV-1 infection (CD4+ T cells, monocytes/macrophages and dendritic cells) that originate from these transduced pluripotent progenitor cells will express the antiviral RNAi constructs and thus prevent HIV-1 gene expression and virus replication. By that, HIV-1 infected CD4+ T cells evade also the destruction by CD8+ cytotoxic T cells as HIV-1 protein production can trigger viral peptide presentation *via* the MHC class I molecules to cytotoxic T cells.

We and others have previously demonstrated remarkably potent virus inhibition even with a single shRNA, but also observed that HIV-1 quickly escapes from RNAi pressure *via* the selection of mutations in the targeted sequence[8-13]. However, a combination of multiple potent shRNAs provided long-term suppression of HIV-1 replication[14-16]. For several years, we have designed and tested various alternative RNAi strategies against HIV-1. Extended shRNA designs and miRNA-like polycistron transcripts were optimized for the expression of multiple inhibitors, but the use of independent shRNA cassettes turned out to be most efficient[9,14,17-21]. Thus, the goal is to use a lentiviral vector with multiple shRNA cassettes that becomes stably incorporated in the human genome. We therefore designed a battery of shRNA inhibitors and tested these in a variety of *in vitro* and *in vivo* experimental settings to allow the selection of the most potent and safe RNAi antivirals. The top candidates were subsequently chosen for the development of a combinatorial RNAi gene therapy against HIV-1 that will be translated into a clinical trial[16]. Primary safety and efficacy studies were performed in the "Human Immune System" (HIS) mouse model[22,23]. Human CD34+ hematopoietic progenitor cells (hHPC) were transduced *ex vivo* with the lentiviral RNAi expression constructs and injected into immunocompromised newborn mice to monitor cell development and differentiation, shRNA expression, cytotoxicity and efficacy of the therapeutic regimen upon HIV-1 infection[24]. This pre-clinical animal model does closely mimic the anti-HIV gene therapy approach proposed for HIV-infected patients.

Here, we will discuss the numerous criteria and corresponding experimental tests that were used in selecting the optimal shRNA reagents for a combinatorial attack on the HIV-1 RNA genome. Ten distinct selection steps can be envisaged (Figure 1): (1) the basic design of the shRNA gene cassettes; (2 + 3) measurement of the antiviral activity in transiently transfected cells and stably transduced cells; (4) selection of the most conserved HIV-1 target sequences to maximize the number of sensitive viral isolates; (5) testing the viral escape possibilities as a measure of the durability of the therapeutic attack; (6) criteria imposed by the use of a lentiviral vector for delivery of the antiviral shRNA cassettes; (7 + 8) screens for possible adverse effects on cell physiology, both *in vitro* and *in vivo*; (9) target site alterations due to resistance mutations for clinically approved antiretroviral drugs; and (10) the assembly of multiple shRNAs to establish a combinatorial RNAi therapy. Along this selection pathway, which took over 7 years, we tested more than 135 shRNA candidates to end up with three potent and safe shRNAs that will be employed in a gene therapy trial (Table 1).

DESIGN OF shRNA MOLECULES

To identify new and potent shRNAs against HIV-1, different design criteria were applied. In general, the shRNA design was based on the prototype shRNA hairpin transcript published by Brummelkamp in 2002: complementary 19-nucleotide sense and antisense strands, a 9-nucleotide hairpin loop and 3'-UU overhang[4]. The antisense strand of this shRNA design will, upon Dicer processing, form the guide strand that instructs RISC for antiviral attack. The complete shRNA cassette consists of the RNA polymerase III H1 promoter, the shRNA sequence followed by the TTTTT termination signal. The H1 promoter, shRNA and termination signal were designed as synthetic DNA or as restriction fragments and cloned into the pSUPER vector (Figure 2A). This cassette can easily be transferred into the lentiviral vector JS1 (Figure 2B) for generation of stably transduced cells[25]. All shRNAs were checked *in silico* to avoid significant complementarity against cellular mRNAs to prevent putative off-target effects.

Over the years, several sets of shRNA inhibitors were tested in our laboratory. We initially described potent suppression of HIV-1 replication with an shRNA that targets nef gene sequences, but viral escape was appar-

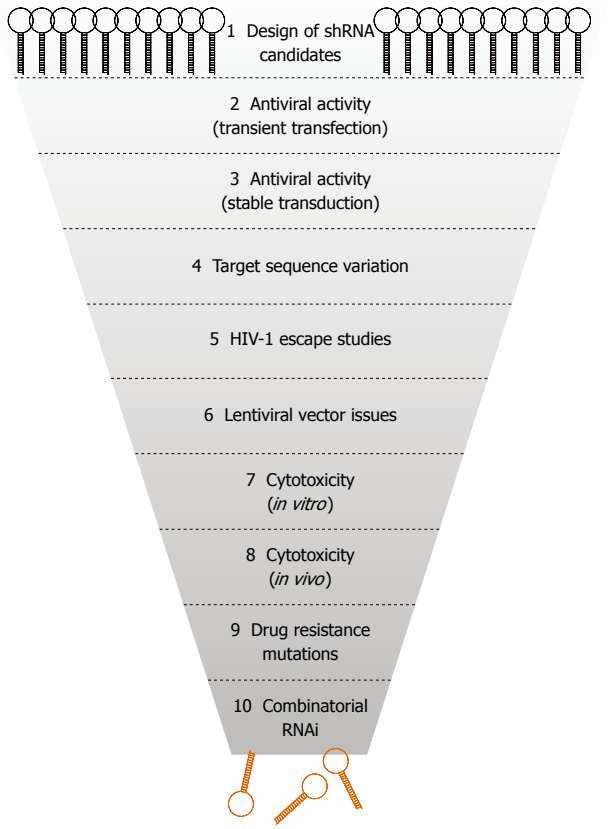


Figure 1 Selection of short hairpin RNAs against human immunodeficiency virus type 1. Scheme of the 10 steps used for the selection of the best shRNA inhibitors for development of an antiviral RNA interference (RNAi) gene therapy. HIV-1: Human immunodeficiency virus type 1.

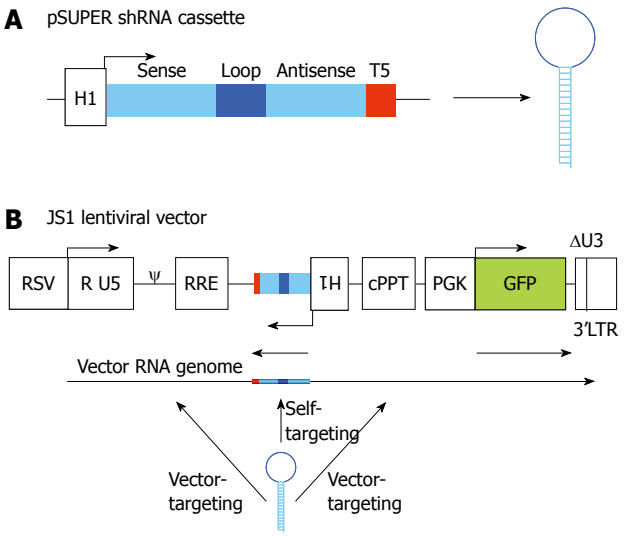


Figure 2 RNA interference vector constructs. A: Two complementary DNA oligonucleotides are annealed and cloned into pSUPER downstream of the H1 promoter that triggers short hairpin RNA (shRNA) expression. The shRNA cassette encodes a 19 nt sense strand, 9 nt loop, 19 nt antisense strand and a stretch of 5 T's (T5), which is the termination signal; B: The shRNA cassette was cloned in the lentiviral vector JS1 for stable transduction of human T cells. The shRNA cassette is cloned in antisense direction to avoid promoter interference. During vector production three transcripts are produced from the lentiviral vector: the shRNA, the vector RNA genome and the GFP transcript. The shRNA will have a 100% target match with the shRNA-encoding sequence in the vector RNA genome (self-targeting), and potential targets in the human immunodeficiency virus type 1 (HIV-1) derived sequences of the lentiviral vector (vector-targeting). RSV: Respiratorial syncytial virus promoter; R U5: R and U5 element of HIV-1 promoter; ψ: Packaging signal; RRE: Rev responsive element; cPPT: Central polypurine tract; PGK GFP: Green fluorescent protein driven from a PGK promoter; 3'LTR: 3' long terminal repeat of HIV-1, with deletion in the U3 region.

ent in prolonged cultures[9,13]. We next tested a first set of 86 antiviral shRNAs that were selected based solely on the conservedness of the target sequence among HIV-1 isolates[14]. Due to the high variability of HIV-1, this selection criterion has become very important for the development of a gene therapy that applies to a broad range of isolates. Our initial studies also revealed the importance of taking the target RNA structure into account for shRNA design as occluded targets are poorly recognized by the RNAi machinery[13,26]. Therefore, we generated a second set of shRNAs that targets particularly accessible regions of the HIV-1 RNA genome based on the SHAPE determined RNA structure model[27,28].

ANTIVIRAL ACTIVITY IN TRANSIENTLY TRANSFECTED CELLS

To evaluate the potency of the shRNAs in terms of anti-HIV activity, we developed a test to measure the inhibition of HIV-1 protein production[14]. For that reason, 293T cells were co-transfected with the HIV-1 molecular clone pLAI, the pSUPER-shRNA vector and the pRL Renilla vector to control for the transfection efficiency. These transfected 293T cells produce infectious virus but do not allow new rounds of infection due to the absence of relevant receptors for HIV-1 attachment and entry. At 48 h post-transfection, the HIV-1 capsid protein (CA-p24) production and Renilla production were quantified. CA-p24 can easily be measured *via* CA-p24 ELISA in the culture supernatant. Then, CA-p24 levels normalized for Renilla expression were compared to virus production with the empty pSUPER control plasmid obtained in co-transfections[14]. Figure 3 indicates the target sites for the most potent RNAi inhibitors plotted onto the HIV-1 genome. Of the 135 shRNA candidates, 44 exhibited at least 80% suppression of HIV-1 production. The upper panel depicts the first set, the lower panel marks the target sites for the second shRNA set[14,27]. Table 1 summarizes the characteristics of the 44 shRNAs that exhibit robust inhibition.

ANTIVIRAL ACTIVITY IN STABLY TRANSDUCED T CELLS

Several of the shRNAs that exhibited significant antiviral activity in the transient transfection assay were subsequently tested in stably transduced CD4+ T cells. To do so, the shRNA expression cassettes were cloned into the lentiviral vector JS1 to allow stable transduction of SupT1 T cells (Figure 2B)[11,14,25]. SupT1 is a commonly used CD4+ T cell line that is permissive for HIV-1 infec-

Table 1 Selection of short hairpin RNAs against human immunodeficiency virus type 1

shRNA name	HIV-1 target		HIV-1 inhibition		Target conservation (%)[5]		Viral escape[6]	Lentiviral vector		Cell toxicity[9]		Drug resistance mutation[10]
	Position[1]	Gene	Transient[3]	Stable[4]	Subtype B	All subtypes		Vector targeting[7]	Titer reduction[8]	*In vitro*	*In vivo*	
LDR2	327[2]	Leader	95	++	66	61	ND	+	ND	ND	ND	-
LDR3	328[2]	Leader	94	++	68	61	ND	+	ND	ND	ND	-
LDR4	329[2]	Leader	99	+++	68	61	ND	+	ND	ND	ND	-
LDR5	330[2]	Leader	94	++	68	61	ND	+	ND	ND	ND	-
LDR7	332[2]	Leader	84	-	69	61	ND	+	ND	ND	ND	-
LDR8	333[2]	Leader	79	++	69	61	ND	+	ND	ND	ND	-
LDR9	334[2]	Leader	91	+++	70	63	ND	+	ND	ND	ND	-
CA	1032	CA-p24	97	-	87	67	ND	-	-	-	ND	-
Gag5	1365	CA-p24	86	++	81	80	+	-	+	+	+	-
Pol1[11]	1910[2]	Prot[11]	97[11]	+++[11]	89[11]	85[11]	+[11]	-[11]	-[11]	-[11]	-[11]	D30N, V32I[11]
Pol2	1911[2]	Prot	86	-	89	85	ND	-	ND	ND	ND	D30N, V32I
Pro 1	1912[2]	Prot	99	+++	85	81	ND	-	-	-	ND	D30N, V32I
Pro 2	1913[2]	Prot	99	ND	80	79	ND	-	ND	ND	ND	D30N, V32I
Pro 3	1914[2]	Prot	99	ND	80	79	ND	-	ND	ND	ND	D30N, V32I
Pro 4	1915[2]	Prot	98	ND	77	76	ND	-	ND	ND	ND	D30N, V32I, L33F
Pro 5	1916[2]	Prot	97	ND	79	78	ND	-	ND	ND	ND	D30N, V32I, L33F
Pro 6	1918[2]	Prot	98	ND	78	77	ND	-	ND	ND	ND	D30N, V32I, L33F
Pro 7	1919[2]	Prot	98	ND	77	76	ND	-	ND	ND	ND	D30N, V32I, L33F
Pro 8	2026	RT	87	ND	58	17	ND	-	ND	ND	ND	D30N, V32I, L33F
Pol6	3755[2]	RT	97	-	72	75	ND	-	ND	ND	ND	-
RT 1 (A)	3757[2]	RT	95	++	71	74	ND	-	-	-	ND	-
RT 2 (B)	3758[2]	RT	98	++	73	75	ND	-	-	+	ND	-
RT 3 (C)	3759[2]	RT	95	++	73	75	ND	-	-	-	ND	-
RT 4 (F)	3760[2]	RT	85	++	69	69	ND	-	ND	ND	ND	-
RT 5 (G)	3762[2]	RT	94	-	72	72	ND	-	-	-	ND	-
Int 1	4310	Int	91	ND	71	19	ND	-	ND	ND	ND	-
Int 2	4344	Int	96	+++	67	25	ND	+	+	-	ND	-
Pol29	4393	Int	82	-	80	80	ND	+	ND	ND	ND	-
Int 3	4420[2]	Int	95	+++	68	61	ND	+	+	+	ND	-
Int 4	4422[2]	Int	99	ND	71	64	ND	+	ND	ND	ND	-
Int 5	4491	Int	81	ND	70	33	ND	-	ND	ND	ND	-
Pol45	4543[2]	Int	92	++	91	75	ND	-	ND	ND	ND	-
Pol47[11]	4545[2]	Int[11]	92[11]	+++[11]	91[11]	73[11]	+[11]	-[11]	-[11]	-[11]	-[11]	-[11]
Vif	4646	Int/Vif	96	-	82	31	ND	-	-	-	ND	-
R/T5[11]	5551	Rev/Tat[11]	93[11]	+++[11]	87[11]	73[11]	+[11]	-[11]	-[11]	-[11]	-[11]	-[11]
Env 1	7250	gp120	81	ND	83	76	ND	+	ND	ND	ND	-
Env 2	7875[2]	gp41	83	ND	49	33	ND	-	ND	ND	ND	-
Env 3	7881[2]	gp41	89	ND	19	6	ND	+	ND	ND	ND	-
Env 4	7884[2]	gp41	85	ND	21	6	ND	+	ND	ND	ND	-
Env 5	8026	gp41	96	+++	53	14	ND	+	+	+	ND	-
Env 6	8277[2]	gp41	98	++	53	18	ND	-	-	+	ND	-
Env 7	8278[2]	gp41	98	++	56	18	ND	-	-	-	ND	-
Env 8	8359	gp41	97	+++	3	1	ND	-	-	-	ND	-
LTR	9072	3′LTR	95	++	53	1	ND	-	-	-	ND	-

[1]Position in human immunodeficiency virus type 1 (HIV-1) LAI mRNA; [2]Overlapping short hairpin RNA (shRNA) clusters; [3]Percentage of inhibition of HIV-1 production in co-transfected cells; [4]Inhibition of HIV-1 replication in stably transduced cells: +++ = strong, ++ = medium, + = low, - = no inhibition; [5]Percentage of sequences in Los Alamos database identical to shRNA target sequence; [6]Detection of escape mutations after prolonged culturing; [7]100% complementarity of the shRNA to JS1 lentiviral vector; [8]Titers compared to JS1 lentiviral vector; + = reduction > 1 log, - = reduction < 1 log; [9]Effects on cell growth; + = negative effect, - = no effect; [10]Drug resistance mutations in the shRNA target region; [11]Selected for the combinatorial gene therapy. ND: Not determined.

tion and that shows clear cytopathic effects (syncytia) upon virus replication. The cells were transduced at a low multiplicity of infection of 0.15 to assure that maximally a single copy of the lentiviral vector integrates per cell. The expression of a GFP reporter gene by the JS1 lentiviral vector allows the easy separation of transduced from non-transduced cells by fluorescence activated cell sorting. The cells are usually sorted 2 d after transduction to obtain a pure GFP-positive population. The transduced cells can subsequently be challenged with HIV-1 and virus replication can be monitored. Infected cultures were inspected on a daily basis under the microscope to monitor cytopathic effects and supernatants were collected to measure CA-p24 production (Figure 4A). SupT1 cells transduced with the empty JS1 vector served as control cells to measure uninhibited viral spread. For future gene therapy applications, shRNAs were only considered if they conferred strong HIV-1 inhibition in transient co-

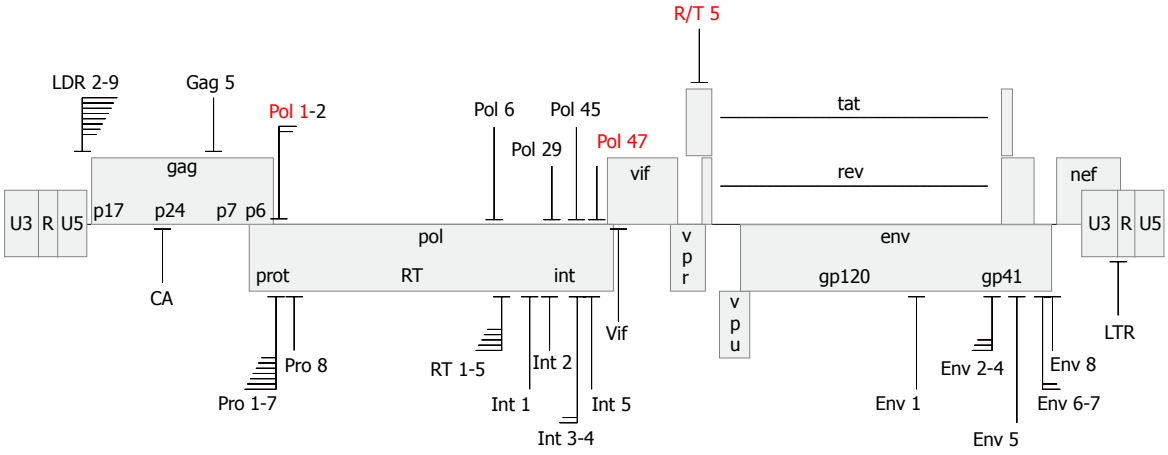


Figure 3 Target sites of anti-human immunodeficiency virus short hairpin RNAs. Depicted is the human immunodeficiency virus type 1 (HIV-1) LAI proviral genome with short hairpin RNA (shRNA) target sites that yielded > 80% suppression of HIV-1 production. Several clusters of overlapping shRNA targets are indicated. The shRNAs have been designed based on conservedness of the target sequence (upper panel) or accessibility in the structured HIV-1 RNA genome (lower panel). shRNAs selected for the R3 combinatorial RNA interference vector are marked in red.

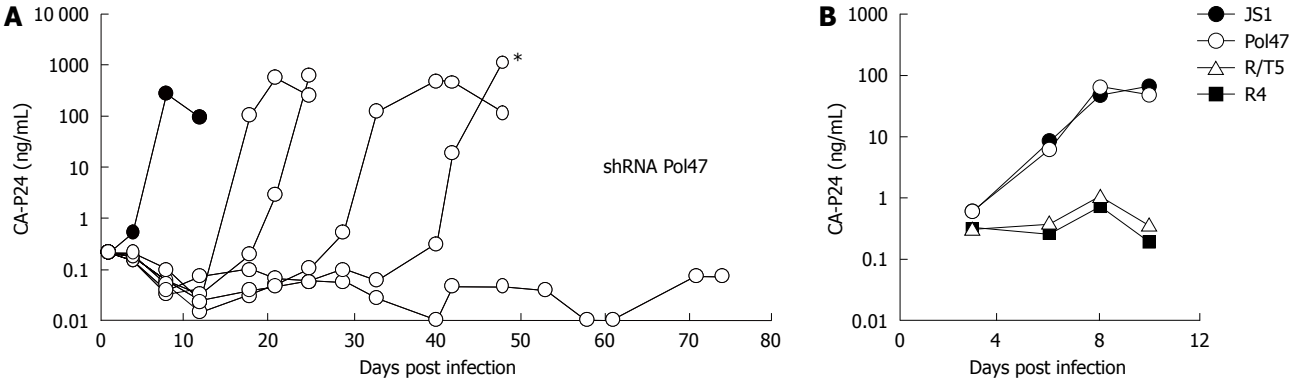
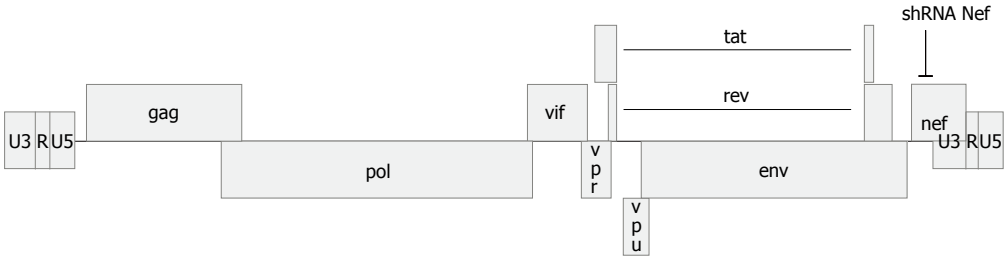


Figure 4 Long-term short hairpin RNA activity in human immunodeficiency virus type 1 replication studies. A: SupT1 T cells expressing short hairpin RNA (shRNA) Pol47 were infected with HIV-1 and virus replication was monitored in five cultures by measuring CA-p24 production for up to 75 d. Cells transduced with the empty lentiviral vector JS1 served as control (dark circles); B: Supernatant from the indicated culture (asterisk in panel A) was passaged on new cells to test the escape phenotype. The virus replicated on control and shRNA Pol47 cells, but not on cells that express another antiviral shRNA (R/T5) or the R4 construct. Adapted from[16].

transfections and stably transduced T cells. In Table 1, this is indicated as "+++" in the respective column.

HIV-1 TARGET SEQUENCE VARIABILITY

Due to the high variability of HIV-1, it is especially important for an anti-HIV gene therapy to target sequences that are relatively conserved among different virus isolates and several HIV-1 subtypes. For most shRNAs, this was an important selection criterion and Table 1 provides an overview of the conservedness of the shRNA targets, both for subtype B that is most prevalent in the Western world and the other subtypes that belong to the HIV-1 group M. To determine the degree of conservedness of the siRNA target sequences, all HIV-1 genome sequences present in the Los Alamos National Laboratory database (http://www.hiv.lanl.gov/) were aligned. The alignment provides the percentage of sequences that are fully complementary to the siRNA for the entire group M and also per subtype. To ensure high sequence conservation, minimally 70% of the viral sequences of each target region have to form a perfect match with the siRNA[14]. This was important for the group M sequences that comprise all subtypes, and specifically the subtype B sequences that are most prevalent in the Western world (Table 1). This standard ensures targeting of a broad spectrum of viral isolates and also decreases the risk of rapid viral escape because mutations of well-conserved HIV-1 sequences are more likely to cause a loss of viral replication efficiency[9-11,13,29,30]. As a current standard diagnostic procedure, the patient-derived HIV-1 sequences of the pol gene are genotyped, including the target sequences for the shRNA inhibitors Pol1 and Pol47. Thus, one will be able to confirm the conservation, such that a full match with the shRNA is guaranteed. Genotyping will also reveal the presence of non-B subtypes, exotic HIV-1 strains and even super-infections that may complicate the RNAi gene therapy[31].



Cultures	Sampling day	shNef target 164 182	
wt		GCTGCTTGTGCCTGGCTAGAAGCACAAG	
A	43	----------	106-nt deletion
B	46	GCTGCTTGT----------AGCACAAG	
C1	27	GCTGCTTGTGCCTGGCGAGAAGCACAAG	
C2	62	GCTGCTTGTACCTGGCGAGAAGCACAAG	
D	62	GCTGCT------TGGCTAGAAGCACAAG	
E	62	GCTGCTTGTGCCTGGCTAGAAG------	63-nt deletion
F	80	GCTGCTTGTGCCTGGCTAGAGGCACAAG	
G	77	----------	225-nt deletion
H	74	ACTGCTTGTGCCTGGCTAGAAGCACAAG	
I	61	GCTGCTTGTGCCTGGATAGAAGCACAAG	

Figure 5 Human immunodeficiency virus type 1 escapes from short hairpin RNA Nef. The human immunodeficiency virus type 1 (HIV-1) LAI proviral genome and the short hairpin RNA (shRNA) Nef target site are indicated. SupT1 cells expressing shRNA Nef were infected with HIV-1 and passaged twice a week until viral escape occurred. Nine different cultures were examined in parallel and the day of sampling is indicated. Part of the nef gene is shown with the shRNA Nef target site highlighted in gray. Numbers refer to the nucleotide position in the nef gene. Escape was apparent by (1) one or more escape mutations in the target sequence; (2) mutations outside the target region; and (3) complete or partial deletions of the target region. Mutations are underlined. Adapted from[13].

HIV-1 ESCAPE STUDIES

Viral escape from the shRNA pressure can occur similar to what is observed under antiviral therapy with antiviral drugs when the HIV-1 target sequence accumulates one or multiple mutations. Extensive viral escape studies have been performed for some shRNAs[8-11,13,32-34]. Transduced and GFP-sorted SupT1 cells were challenged with a high amount of virus and passaged over time. When viral outgrowth was observed, the cell-free supernatant was transferred to a new culture of shRNA-expressing cells to confirm the resistance phenotype (Figure 4A and B). Cellular DNA with the integrated proviral genome can subsequently be isolated, the siRNA target region can be PCR-amplified and cloned into a plasmid for sequence analysis[11,13,16,35]. At this point, it is important to filter out 'pseudo-escape' events that are due to breakthrough virus replication when a high virus input is tested, often in combination with a sub-optimal inhibitory shRNA regimen. In this scenario, the pseudo-escape virus can be recognized because it will not carry any resistance mutation and will obviously lack the resistance phenotype[9,11,16].

We routinely test multiple HIV-1 evolution cultures in parallel because viral evolution is a chance process driven by randomly occurring mutations, some of which are beneficial and thus subsequently selected under RNAi pressure. For example, the diverse viral escape routes observed in independent cultures of shRNA Nef expressing cells are depicted in Figure 5[13]. We reported three types of HIV-1 escape: (1) Mutation(s) in the siRNA target sequence; (2) a mutation in the flanking region that influences the local RNA structure; and (3) partial or even complete deletion of the target sequence. The latter escape route seems possible only in case non-essential viral sequences are targeted. Indeed, deletion-based viral escape was never witnessed when essential HIV-1 sequences encoding the Protease and Integrase enzymes were targeted[10,32]. This observation supports the notion to target well-conserved viral sequences that usually encode the more essential viral functions. It must be pointed out that viral escape studies are extremely time and labor-intensive. Therefore, such investigations should only be conducted for candidate shRNAs that fulfill multiple criteria, e.g., potent inhibition in transient transfections and stably transduced T cell infections.

LENTIVIRAL VECTOR CONSIDERATIONS

HIV-1 causes a persistent infection in humans, which requires durable expression of the inhibitory shRNAs. Therefore, the use of a lentiviral vector seems ideal because of its property to stably integrate into the host cell genome, which allows a constant supply of antiviral shRNAs. The third generation lentiviral vectors have proven to be safe for use in humans and no insertional oncogenesis has been reported thus far[36-38]. These vectors transduce dividing and non-dividing cells and can thus be applied, e.g., in hematopoietic progenitor cells[23,39-43]. For clinical application, it is important that the vector can be produced to high titers. We and others previously report-

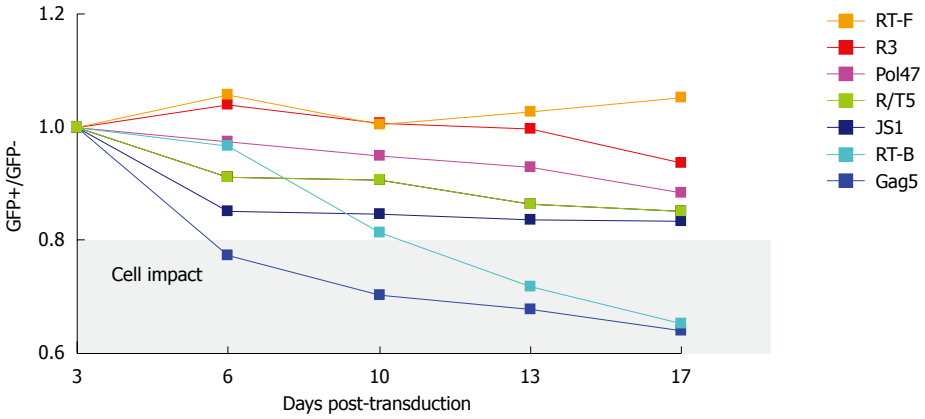


Figure 6 Competitive cell growth assay. SupT1 T cells were transduced with a short hairpin RNA (shRNA)-expressing lentiviral vector, yielding a cell population with approximately 40% GFP-positive cells. The ratio of GFP-positive cells at day 3 after transduction was set at 1 and measured longitudinally. The cells were passaged and analyzed *via* FACS measurement twice a week. JS1 represents the empty lentiviral vector without shRNA expression. The gray window highlights shRNAs that have a significant adverse effect on cell growth.

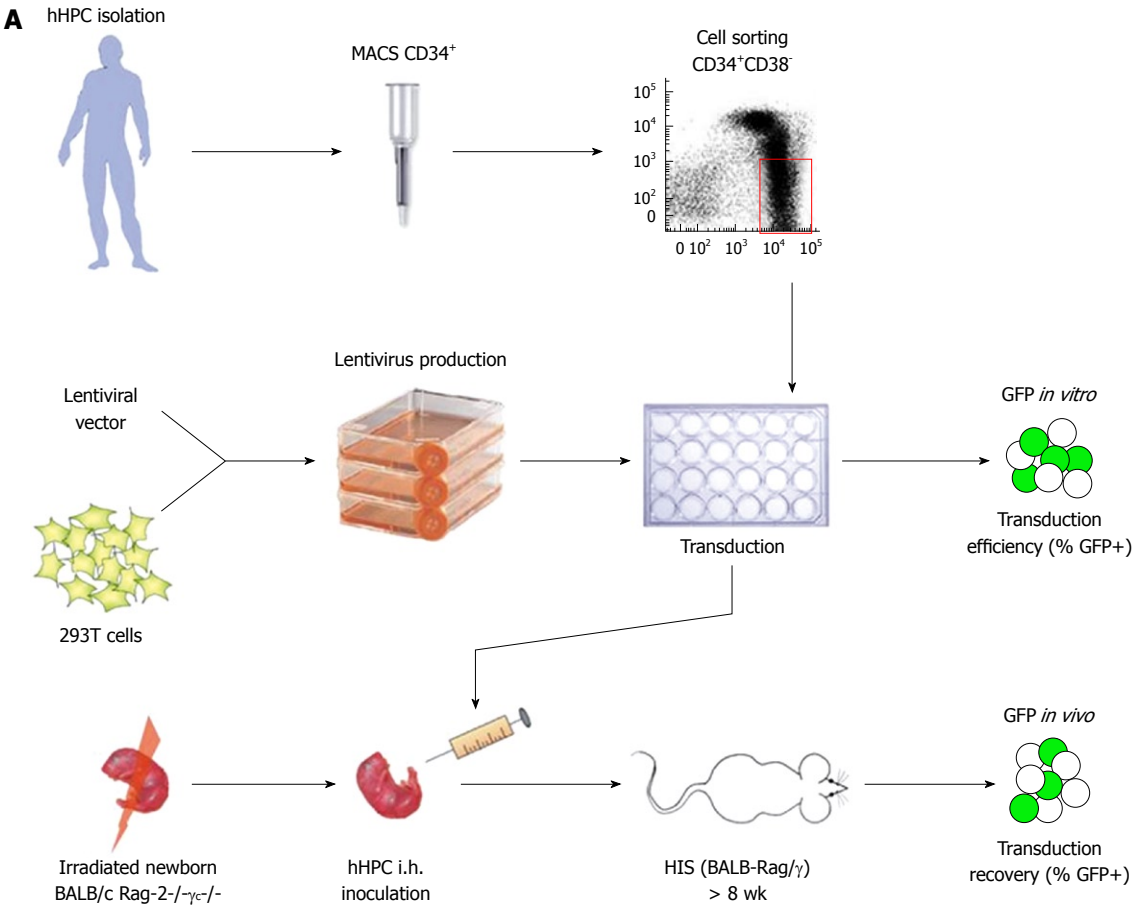
ed titer problems with lentiviral vectors encoding antiviral shRNAs that may obstruct clinical application[44-48]. Lentiviral vectors are produced by co-transfection of the lentiviral vector construct JS1 (Figure 2B), Gag-Pol and Rev-expression plasmids, and a VSV-G envelope construct. During lentiviral vector production, all the different mRNAs are expressed in the producer cell together with the shRNA transcript. The vector transcript does in fact include the shRNA sequence and will thus have a perfect target for siRNA-mediated degradation. However, such self-targeting does not easily occur because the target is occluded in a stable shRNA hairpin structure and therefore protected from RNAi attack. Further complications arise when the shRNA targets HIV-1 derived sequences in one of the lentiviral vector constructs. This is referred to as vector targeting. We previously discussed in detail all possible routes by which shRNAs could impede lentiviral vector production and how to prevent or overcome these specific problems[45,48]. Of course, acute cytotoxicity of the expressed shRNA can also cause a serious titer reduction due to effects on the producer cell viability and this may eventually also affect the viability of transduced cells, i.e., the gene therapy target cells.

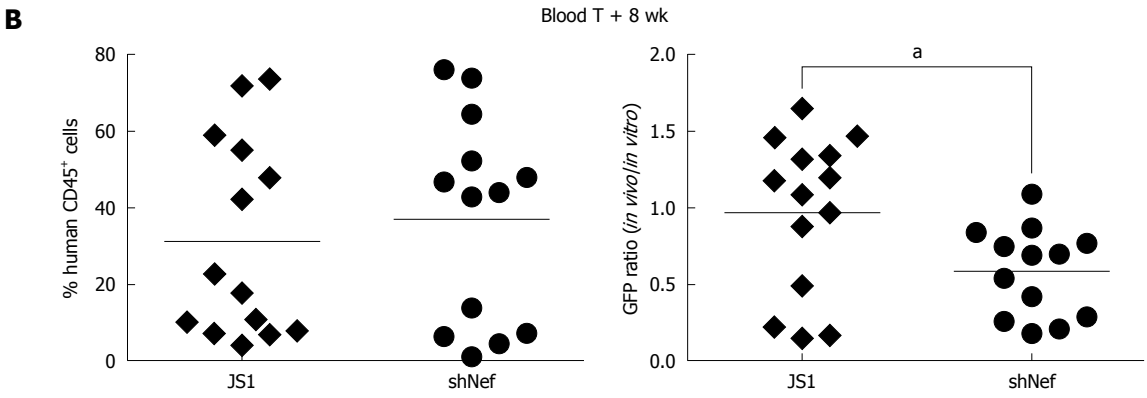
CYTOTOXICITY IN *IN VITRO* CELL CULTURE

The antiviral shRNA may exhibit adverse effects on cell growth through silencing of cellular mRNAs (off-target effects) or saturation of the RNAi machinery, in particular when the shRNA is overexpressed[49]. These effects cannot easily be predicted and should thus be tested experimentally. There are several ways to score the impact of shRNA expression on cell viability and physiology. One could for instance determine the cellular doubling time by frequent cell counting. We recently developed a very user-friendly and ultra-sensitive assay that follows over time the ratio of shRNA-expressing GFP-positive cells *vs* untransduced GFP-negative cells in a co-culture assay[50]. This competitive cell growth or CCG assay has some clear advantages over other well-established cell proliferation assays: (1) After cell transduction, only a small aliquot of the culture is needed to launch the CCG assay, without any extra steps; (2) The CCG assay is internally controlled as it starts with a mixture of transduced and untransduced cells; and (3) Even minor effects on the cellular proliferation rate caused by shRNA expression can be detected. We screened all promising shRNAs in this assay (Figure 6). Besides single shRNA-expressing vectors, we also investigated combinatorial vectors such as R4 (Gag5, Pol1, Pol47, and R/T5) and R3 (Pol1, Pol47, and R/T5). shRNAs that exhibit negative effects on cell growth such as Gag5 should be excluded from combinatorial RNAi vectors (Figure 6, Table 1). Removal of Gag5 from the R4 vector that exhibited impaired cell proliferation led to the design of the R3 lentiviral vector that scored no negative cell growth effects. Cytotoxicity by saturation of the cellular RNAi pathway is especially critical for the combinatorial shRNA vectors and might have contributed to the adverse R4 effects.

CYTOTOXICITY *IN VIVO*

Before one can proceed to a gene therapy trial in humans, safety should ideally be demonstrated in a preclinical animal model. As the RNAi mechanism is based on perfect sequence complementarities between siRNA and the viral target, the simian immunodeficiency virus/macaque model cannot be used for such studies. However, mice with the complete human immune system were created by injection of hHPCs into immunodeficient (BALB/c Rag2-/- IL2Rγc-/-) newborn mice. All major subsets of the human innate and adaptive immune system are found in the reconstituted HIS mice[22,23]. This HIS mouse model is ideally suited to test our gene therapy for several reasons. First, the hHPCs that are engrafted in the Rag-2-/-γc-/- newborn mice (Figure 7A) are similar to the ones that we propose to modify in our *ex vivo* gene therapy of





	GFP-				GFP+			
	T cells	4/8 ratio	B cells	Non T/B	T cells	4/8 ratio	B cells	Non T/B
JS1	26.5 ± 9.3	0.74 ± 0.23	60.2 ± 9.0	13.3 ± 2.1	21.9 ± 9.1	0.90 ± 0.26	56.2 ± 7.5	21.8 ± 4.1
shNef	20.8 ± 9.1	0.71 ± 0.11	67.8 ± 8.8	11.4 ± 2.2	18.2 ± 7.7	0.94 ± 0.21	55.1 ± 7.7	26.7 ± 5.0

Figure 7 ***In vivo*** **safety studies in the HIS mouse model.** A: Cell suspensions enriched for human hematopoietic progenitor cells (hHPC) are prepared from fetal liver tissue. Live nucleated CD34+ cells are magnetically sorted and further enriched for the hHPC (CD34+CD38- fraction) using fluorescence activated cell sorting. Lentiviral supernatants are produced on 293T cells. hHPC are transduced *ex vivo* with the shRNA-expressing lentiviral vector and injected intrahepatically into sub-lethally irradiated newborn BALB/c Rag-2-/-γc-/- mice. The transduction efficiency is evaluated based on GFP expression after 3.5 d in culture (GFP *in vitro*); B: The HIS (BALB-Rag/γ) mice are analyzed in the blood and the organs after at least 8 wk post-transplantation for the presence of human cells (%CD45+ cells) (left graph), which were analyzed for GFP recovery (right graph). The GFP recovery is the ratio between the frequency of human GFP+ cells measured in the animals (GFP *in vivo*) and the frequency of GFP+ hHPC injected in the newborn mice (GFP *in vitro*, transduction efficiency). The major subsets of the human immune system in the blood are also analyzed for their frequency and absolute number in the human GFP+ and GFP- population. Adapted from[24,70]. [a]$P < 0.05$.

HIV-1 seropositive patients. Second, as the hHPCs transplanted in the mice consist of a mixture of transduced (GFP+) and non-transduced hHPCs (GFP-), the human immune system in the animals will thus be constituted by transduced shRNA-expressing cells (GFP+) and non-transduced cells (GFP-). This provides an internal con-

trol to test for adverse effects of shRNA expression on hematopoiesis. Finally, as the HIS mouse can be infected by HIV-1, both the safety and efficacy of the shRNA therapy can be evaluated in the HIS mouse model.

The safety of an shRNA is assessed in the blood and the organs of the animals by multiple factors: (1) The presence of the human hematopoiesis-derived CD45+ GFP+ and GFP- cells; (2) the ratio between the frequency of human GFP+ cells measured in the animals and the frequency of human GFP+CD34+ cells injected in the newborn mice; and (3) the frequency and absolute number of different cell subsets of the human immune system, such as CD4+ and CD8+ T cells, B cells, monocytes and dentritic cells (Figure 7B). We previously tested the feasibility of an shRNA-based gene therapy in HIS mice reconstituted with hHPC transduced with a lentiviral vector expressing an shRNA against the HIV-1 nef gene[24]. In this model hHPC expressing anti-HIV shRNAs give rise to multi-lineage reconstitution of the human immune system *in vivo* and generate CD4+ T cells with the ability to resist HIV-1 replication in a sequence-specific manner. We tested our four candidate shRNAs and observed normal development of the human immune system in the animals for three of these shRNAs (Centlivre *et al* manuscript in preparation). A negative impact of Gag5 on the hematopoiesis of the HIS mouse was scored, confirming the *in vitro* findings in the CCG assay. These combined results led to the exclusion of Gag5 from the combination gene therapy (Table 1). The three "safe" shRNAs will now be combined into a single lentiviral vector for further *in vivo* safety tests.

PRE-EXISTING DRUG RESISTANCE MUTATIONS

Our proposed anti-HIV gene therapy will be developed for therapy-experienced HIV-1 infected individuals who have failed on regular antiretroviral drug regimens. As drug resistance mutations may affect the viral genome sequences targeted by RNAi, we investigated whether the target sequences of the top shRNA candidates are likely to acquire drug-resistance causing mutations. For this, we screened the Stanford HIV-1 drug resistance database[51,52]. The relevant drug resistance substitutions in the inhibited viral proteins are plotted in Table 1. In particular, the Protease gene sequence targeted by the set of overlapping shRNAs (Pol1-2, Pro1-7) has been implicated in the acquisition of resistance against Protease inhibitors like Nelfinavir, Aprenavir, Ritonavir and Indinavir at codons 30, 32 and 33. A treatment history that includes one of these Protease inhibitors and genotyping results that demonstrate the presence of at least one of these mutations will be an exclusion criterion for gene therapy participants.

COMBINATORIAL RNAi

The stable expression of anti-HIV shRNAs in T cells results in potent virus inhibition[16,53,54]. However, the application of a single shRNA inhibitor is not sufficient to maintain inhibition. Virus escape variants can emerge after extensive culturing[8-11,13,34]. Therefore, multiple antiviral shRNAs should be expressed simultaneously to achieve durable inhibition by raising the genetic threshold for viral escape[48,55,56]. This combinatorial strategy is analogous to current antiretroviral therapy regimens with multiple drugs that have led to significant clinical success in HIV-1 infected patients[57].

There are several ways to express multiple RNAi inhibitors against HIV-1, ranging from polycistronic miRNAs to extended multimeric shRNA transcripts[17-21]. We achieved most promising results with multiple shRNA cassettes[14,16]. To express multiple shRNAs, we initially inserted several H1-driven shRNA expression cassettes into the lentiviral vector. However, these vectors are extremely unstable as shRNA cassettes were deleted during transduction due to slippage of the Reverse Transcriptase enzyme on the repeated H1 promoter sequences[16,58]. To prevent recombination-mediated deletion of shRNA cassettes, we designed shRNA cassettes with unique promoter elements. The RNA polymerase III promoters H1, U6 and 7SK and the RNA polymerase II promoter U1 were used[4,59-61]. All these promoters have precise transcription start and termination sites and the shRNA expression levels are similar. The combination of four promoter-shRNA cassettes in R4 (U1-R/T5, U6-Pol1, 7SK-Gag5, and H1-Pol47) leads to durable virus inhibition in stably transduced T cells[16]. At present, the R4 combinatorial RNAi vector has been modified to R3 without Gag5 due to adverse cellular effects of this shRNA. The R3 lentiviral vector that confers the same potent and durable inhibition is proposed for the future clinical anti-HIV gene therapy trial.

CONCLUSION

We describe here the course that was taken to select the most potent and safe shRNA inhibitors against HIV-1, which will contribute to the development of an exclusively shRNA-based gene therapy against HIV-1. Currently, the combinatorial RNAi approach comprises three shRNAs targeting three distinct and highly conserved regions of the HIV-1 RNA genome.

The proposed RNAi gene therapy against HIV-1 will be developed for therapy-experienced HIV-1 infected individuals. During antiretroviral therapy, mutations can be selected in the genes that encode the drug-targeted viral proteins. Such mutations can interfere with RNAi attack when siRNA target sites are altered. Indeed, one shRNA inhibitor of the combinatorial shRNA vector targets a viral sequence that frequently acquires mutations to escape from Protease inhibitors. Patients who failed on Protease inhibitor containing regimens or that harbor viruses with such resistance mutations have to be excluded from the current combinatorial RNAi gene therapy. To overcome this issue, alternative shRNA regimens could be established that do not target viral genome regions

known to acquire prominent drug resistance mutations. Alternatively, one could attack the resistant HIV-1 strains with modified shRNA inhibitors. We previously showed that a combination shRNA strategy directed against the wildtype and drug-escape variants was able to efficiently and durably suppress virus replication[35,48].

The selection of HIV-1 escape variants must be prevented to durably suppress the chronic virus infection. To achieve this, targeting of conserved HIV-1 RNA regions is important, as well as the simultaneous application of multiple shRNAs. Potent virus inhibition will reduce the chance of virus escape by limiting the occurrence of mutations and the genetic threshold for resistance development is increased when multiple viral sequences are targeted. We continuously work on improvement of the shRNA design and recently identified loop sequences such as miRNA-derived loops that improve the siRNA processing and yield more potent gene knockdown and HIV-1 inhibition[62]. Alternatively, targeting of cellular cofactors that are essential for HIV-1 replication represents a promising anti-escape approach. The mutation rate of the cellular DNA replication machinery is significantly lower than that of the lentiviral Reverse Transcriptase enzyme. Thus, the chance that resistance mutations are selected in host mRNAs is negligible compared to HIV-1 target sequences. Recent screens revealed several cofactor-encoding mRNAs whose knock-down resulted in diminished HIV-1 replication[63-67]. However, knockdown of cellular proteins at the mRNA level might have negative effects on cell viability and anti-host shRNAs must be carefully designed. An ideal candidate cofactor is the CCR5 co-receptor. A natural deletion in the CCR5 gene (CCR5-Δ32) has been found at 1% frequency in the Caucasian population. These individuals are resistant to HIV-1 infection and do not appear to suffer from major biological effects or health issues due to the absence of this receptor protein[68].

Within a couple of years, RNAi has moved from the laboratory to clinical trials as novel therapeutic against a variety of diseases. In 2008, the first antiviral shRNA was used in combination with a TAR decoy and CCR5-ribozyme as an RNA-based gene therapy for HIV-1 infected individuals. The transfused cells were successfully engrafted and the anti-tat/rev siRNA was detected in peripheral blood mononuclear cells (PBMCs) up to 24 mo[69]. This initial clinical result provides encouragement for the anti-HIV gene therapy that we develop based exclusively on multiple shRNAs. The extensive preclinical assays in the humanized mouse model demonstrated the safety and efficacy of this combinatorial RNAi approach, which will soon move towards clinical testing.

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REVIEW

Innate and adaptive immune responses against picornaviruses and their counteractions: An overview

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Abstract

Picornaviruses, small positive-stranded RNA viruses, cause a wide range of diseases which is based on their differential tissue and cell type tropisms. This diversity is reflected by the immune responses, both innate and adaptive, induced after infection, and the subsequent interactions of the viruses with the immune system. The defense mechanisms of the host and the countermeasures of the virus significantly contribute to the pathogenesis of the infections. Important human pathogens are poliovirus, coxsackievirus, human rhinovirus and hepatitis A virus. These viruses are the best-studied members of the family, and in this review we want to present the major aspects of the reciprocal effects between the immune system and these viruses.

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Key words: Picornaviruses and infection; Poliovirus; Coxsackievirus; Human rhinovirus; Hepatitis A virus; Adaptive immune system; Innate immune system; Antibody response and inflammation; Apoptosis; Interferon and cytokines

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INTRODUCTION

The picornavirus family represents a diverse group of viruses that are classified within 12 genera[1] (Table 1). These viruses cause a variety of human and animal diseases, including the common cold [human rhinoviruses (HRV)], myocarditis [coxsackie viruses (CV)], hepatitis [hepatitis A virus (HAV)] and poliomyelitis [poliovirus (PV)]. Because of their clinical relevance, these human pathogens, which except the hepatovirus HAV are members of the enterovirus genus, are the best-studied members of the family[2-5]. Although many details about the replication of these viruses are known[6,7] the pathogenesis of the heterogeneous clinical appearances and manifestations of the particular diseases, varying between asymptomatic and fatal, is poorly understood, but is closely linked to the immune responses induced after infection. Therefore, knowledge of the specific immunological activities following the entry of these viruses into the human host will provide the basis for a better understanding of the pathogenic processes.

The spherical, nonenveloped virions of picornaviruses range in diameter between 27-30 nm. The genome is a positive-strand RNA of 7000 to 9000 nucleotides covalently linked at the 5' end to the viral protein 3B (VPg) and is translated cap-independently by internal ribosomal entry into a polyprotein (VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-3C-3D), which yields the eleven proteins through various independently functioning intermediates, upon cleavage by viral proteases. Replication occurs in the cytoplasm in association with intracellular membranes. Picornaviruses are usually considered to be

released from infected cells by cell lysis[8], which applies for the cytopathogenic rhino-, coxsackie- and PVs, but not for HAV[9], for which the release process is not known. However, damage to tissues results not only directly from virus replication, but also from the host response to infection.

The host immune response against picornaviruses is diverse and complex, and this is reflected by the numerous data obtained in studies particularly with PV, CV, RV and HAV, and this review discusses various aspects of the immunology of these viruses.

The host fights virus infections by employing various mechanisms, including cytokine release, antibody production and cytotoxic T cell (CTL) activation. The importance of each mechanism, however, strongly varies according to the virus concerned.

As a large part of the infections with any of these four viruses proceeds asymptomatically, it can be assumed that the innate immune system, which responds within minutes after viral entry into host cells[10], is able to block viral replication to a certain degree. Sensing of specific structures of the viral nucleic acid like double-stranded RNAs, which occur as replicative intermediates and are recognized as pathogen associated molecular patterns, is accomplished in the cytoplasm by Toll-like receptor (TLR)3, which is associated with intracellular vesicles, or by the sensors retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5)[11] resulting in synthesis of cytokines with strong antiviral activity, like type I interferon (IFN). MDA-5, not RIG-I, is believed to be crucial for sensing infection with picornaviruses. This was concluded from studies with mice lacking the mda-5 gene. These mice are deficient in the production of type I IFN in response to the cardiovirus enzephalomyocarditis virus (EMCV), and the animals showed a higher susceptibility to infection[12,13]. But sensing of picornaviral RNA seems to be more complex, and the other sensors might also be involved, as overproduction of RIG-I in cultured cells is also able to reduce EMCV replication[14]. On the other hand, PV, CV, HRV as well as HAV have developed mechanisms to interfere with the signaling from the above mentioned sensors, although in different ways. This ability of the viruses seems to be necessary firstly to establish infection and secondly, to maintain replication for a longer time, as activation of the virus specific adaptive immune response by a specific cytokine mix generated during the innate response is retarded.

After primary infection, antibodies seem in general to be important to control viral viremic spread within the infected tissue as well as to distant further organs, and thus to retard the severity of the disease. Infections with PV and CV seem to be controlled efficiently by antibodies, as prolonged replication of PV occurs in immunodeficient patients[15] and prolonged excretion of coxsackievirus as well as chronic encephalitis after coxsackievirus infection was observed in patients with agammaglobulinemia[16-20]. This is supported by the finding, using $CD4^+$ T-cell deficient mice, that both PV and CV efficiently induce B cells to proliferate and produce IgM independent from T-cell help (TI IgM)[21-24]. This TI antibody response results from extensive B-cell receptor cross linking by the highly organized, repetitive virion structure and is postulated to be a characteristic of antibody-controlled cytolytic viruses[24]. In contrast, HRV seems to be controlled by the innate immune response as the antibody response appears after recovery from illness[25] and HAV seems to be eliminated by CTL[26,27]. Upon infection, these four viruses are expected to induce the production of secretory IgA (SIgA), serum IgA (sIgA) and IgG, which is due to their route of transmission. PV, CV and HAV are transmitted fecal-orally, whereas HRV is transmitted by the respiratory route. But there are significant differences between the viruses in the time courses of the different antibody classes as well as of the antibody responses. For example, the IgG response after infection with HAV is strongly delayed[28,29] and only a weak antibody response against HRV, which is boosted after resolution of the symptoms, develops[25]. It seems at least to be the rule for all four viruses that antibodies are critical to prevent reinfections. Even during asymptomatic courses of the diseases, production of neutralizing antibodies may be induced (occult immunization). In order to counteract this effect, polio- and coxsackieviruses form serotypes (PV: 3 serotypes, CV: 29 serotypes) which are defined as different viral strains that do not elicit cross-neutralizing antibodies. This enables the viruses to evade antibody-controlled reinfections and to infect the same individual several times by a different serotype despite the presence of possibly high titers of neutralizing antibodies and cross-reactive T-cell help against the first serotype. This also seems to apply for rhinovirus, which forms 102 serotypes, despite its weak and apparently delayed antibody response. Only in the case of HAV, which exists as only one serotype, are reinfections efficiently prevented by anti-HAV IgG induced by the first infection.

In the following sections the specific interactions between the different viruses and the immune system will be described.

ADAPTIVE IMMUNE RESPONSE

In general and on average, the adaptive immune response during viral infections is induced 3 to 5 d after the infection occurred, a time point when infections are established and the amount of progeny virus reaches a level required to activate the adaptive response by assistance of cytokines, which are released during the innate immune response. Before the $CD8^+$ T-cell response (CTL) reaches its peak 7 to 10 d after the infection, natural killer cells (NK cells) are present. The T-cell response decreases within 3 to 4 wk, but memory and splenic $CD8^+$ T-cells remain present. Antibodies produced by the adaptive B-cell response are barely detectable in the acute, symptomatic stage of the disease, but increase over a period of 2 to 4 wk. Virus and virus infected cells are normally eliminated 2 wk after infection and serum antibody as well as memory B and T cells remain.

Table 1 Picornavirus family

Genus	Normal host organism	Including for example
Aphtovirus	Cattle, swine	Foot-and-mouth disease virus
Cardiovirus	Humans, small rodents	Encephalomyocarditis virus Saffold virus
Enterovirus	Humans, cattle, swine	Poliovirus Coxsackievirus Rhinovirus
Hepatovirus	Humans	Hepatitis A virus
Parechovirus	Humans, small rodents	Human Parechovirus Ljungan virus
Erbovirus	Horses	Equine rhinitis B virus
Kobuvirus	Cattle	Aichi virus
Teschovirus	Swine	Porcine teschovirus
Sapelovirus	Birds, swine	Avian sapelovirus Porcine sapelovirus
Senecavirus	Swine	Seneca valley virus
Tremovirus	Birds	Avian encephalomyelitis virus
Avihepatovirus	Birds	Duck hepatitis A virus

Some viruses infecting bats (Juruaca virus), fish (Bluegill virus), reptiles, amphibians and ticks (Sikhote Alyn virus, Syr-Dorya Valley fever virus) are not classified within a genus so far. Plant picornaviruses differ from the animal viruses in some properties and have been classified into the family Secoviruses.

Whereas the adaptive immune response to polio- and CV roughly follows the above-described general scheme, the adaptive immune responses to human rhinovirus and HAV significantly deviate from the average course. In general, the adaptive response is required for complete virus clearance, and there is considerable data about the antibody responses to these four viruses (Figure 1) but, with the exception of HAV, little is known about the CTL response, and the role of these T cells is controversially debated. The CTL response against HAV which is the only non-cytopathogenic virus presented here is well investigated and it is shown that the symptoms of hepatitis A can be attributed to an immunopathogenic process caused by the activity of HAV-specific CTLs.

Neutralization of picornaviruses is mediated through antigenic sites, which are conformational, discontinuous and complex structures formed on the surface of the virions by exposed loops between the β-strands of certain structural proteins (structural proteins of picornaviruses building the surface of the capsid are VP1, VP2 and VP3; VP4 lines the inside of the viral particle). PVs have three distinct antigenic sites[30,31], which have been exactly identified. One antigenic site is formed by amino acids of the structural protein VP1 (aa 90-100, 220-223 and 286-290), the second site includes residues from VP1 and VP2 (aa 164-172) and the third site includes residues from VP1 and VP3 (aa 58-60 and 70-80). Within coxsackievirus capsids conformational and linear antigenic sites were found, which include residues from VP1, VP2 and VP3[32-34]. HRV exhibit four different antigenic sites, which are also formed by residues from VP1, VP2 and VP3[35]. HAV holds only one immunodominant antigenic site, which contains amino acid residues contributed by VP1 and VP3[36,37]. Three complementary mechanisms of neutralization are assumed[38]. Firstly, antibodies bound to the virus particle interfere with the attachment to the cellular receptor. Secondly, neutralization is a result of antibody-mediated aggregation of virions, which prevents attachment and uptake of virus, and thirdly, binding of antibodies to separate structural subunits within the capsid structure inhibits uncoating.

In the following, an overview of the adaptive immune responses against PV, CV, HRV and HAV is given.

PV

After fecal-oral transmission, the major site of replication is the intestinal tract (epithelia and Peyer's patches). After a mean incubation time of 7 d, influenza-like symptoms develop, from which the patient recovers within a few days. Fecal excretion of PV occurs shortly after infection and persists for approximately 7 wk, and a short viremic phase appears between 3 to 7 d after infection. Besides this abortive poliomyelitis, nonparalytic poliomyelitis may occur in 1%-2% of the infections with viral invasion of the CNS leading to meningitis and muscle spasm. The illness lasts for approximately 6 d. In up to 2% of the cases paralytic poliomyelitis occurs, and in 80% of these patients residual paralysis persists.

Neutralizing anti-PV IgM antibodies appear 3 d after infection, reach their peak titer after 9 d and disappear in the course of 4 wk[39-41] (Figure 1).

The anti-PV IgG response is also briskly appearing 3-4 d after exposure. These antibodies reach the peak titer 3-4 wk after infection and persist for years, perhaps lifelong[39-44]. The antibodies seem to be responsible for controlling viremia, as the termination of viremia immediately follows the detection of neutralizing antibodies (Figure 1). Already low levels of circulating anti-PV antibody, including passively given immune globulin, are able to prevent the paralytic disease. This indicates that infection of the CNS requires or is at least supported by viral spread through blood, and therefore significantly depends on the velocity and strength of the antibody response[45]. However, virus excretion continues for about 1 mo. Although it is not clear why shedding is going on for so long, the termination of shedding and final viral clearance seem also to be mediated by antibodies because hypogammaglobulinemia may result in persistent excretion for years[46] and because T-cell deficiency does not result in persistent viral excretion[47,48].

Mucosal anti-PV IgA (SIgA) is detectable 1 wk after infection in pharyngeal and stool samples, whereas sIgA appears 3 wk post exposure (Figure 1). Both responses reach their maximum levels approximately 4 wk after infection, but SIgA with a higher magnitude than sIgA[39-41]. The source of these antibodies is not known. The mucosal immunity to PV provides substantial resistance against secondary infections[39], and seems to play an important role in preventing spread of PV. This is evident, as in contrast to inactivated PV vaccine, which is less effective than the live vaccine in stimulating enteric immunity, the

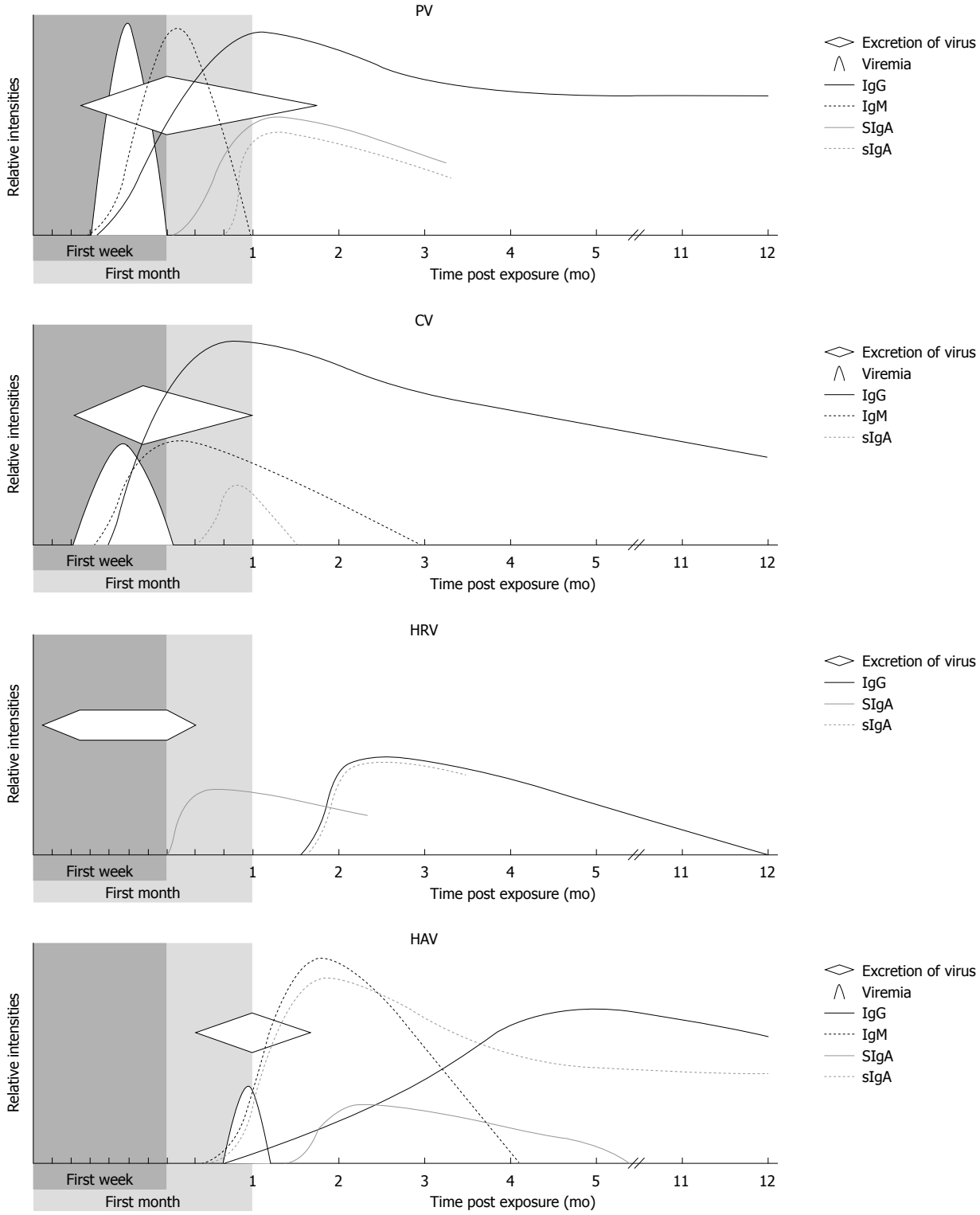

Figure 1 Time courses of viral excretion, viremia and of the antibody responses after infection with poliovirus, coxsackievirus, human rhinovirus and hepatitis A virus. This figure schematically shows the mean duration and relative intensity of viral spread and the antibody responses against the viruses. PV: Poliovirus; CV: Coxsackievirus; HRV: Human rhinovirus; HAV: Hepatitis A virus; SIgA: Secretory IgA; sIgA: Serum IgA.

oral live-attenuated PV vaccine is much more effective in preventing intestinal infection and has a much greater dampening effect on fecal shedding of PV[49,50]. Both vaccines, however, induce similar levels of circulating antibodies. As mentioned above, the mechanism by which anti-PV antibodies terminate viral shedding and are able to clear infection is not known. However, it can be assumed that in the intestinal epithelium the cell-to-cell spread of progeny viruses, which are released after lysis of the infected cells, is interrupted by the enteric anti-PV IgA antibodies[41]. Alternatively, antibody-mediated lysis of infected cells could also be involved.

In contrast to the neutralizing antibody response to PV, much less is known about the adaptive T-cell responses and their probable role in PV infections. PV-specific $CD4^+$ T cells are induced in vaccinated individuals, and epitopes have been identified[51,52]. The induction may occur by dendritic cells and macrophages infected with PV[53]. This also shows that HLA class Ⅱ presentation remains intact in infected antigen presenting cells. The resultant $CD4^+$ T cells were also able to produce IFN-γ and lyse infected target cells[53]. The cytolytic ability together with the ability to secrete IFN-γ allows the assumption that PV-specific $CD4^+$ T cells may play a role in virus clearance. Furthermore, stimulation of PV-specific cytotoxic $CD8^+$ T-cell (CTL) responses by infected macrophages could be demonstrated, and these CTLs secreted IFN-γ[53]. This implies that virus clearance is not only due to the $CD4^+$ T-cell/antibody response, but that the CTL response might also play a role. Since the 1950s, inactivated and live-attenuated PV vaccines have been available[50].

Coxsackie virus

After respiratory and fecal-oral transmission, respectively, the incubation time is approximately 5 d, but may last for up to 35 d. These viruses cause a systemic disease, both acute and chronic, with a wide variety of symptoms. Besides infection of the epithelial tissues, the viruses exhibit myotropism and a tendency to infect the central and peripheral nervous system by humoral spread, and the acute clinical appearances may range from influenza-like symptoms (minor summer illness) to myocarditis, aseptic meningitis as well as myelitis. The duration of acute illness is usually between 3 and 8 d. Post-acute symptoms include myocarditis and pericarditis, which may persist for weeks[54-58] and infections have been linked to the induction of autoimmune diseases such as chronic myocarditis and type 1 diabetes. Excretion in feces and nasal secretions, respectively, occurs between days 2 and 28 after infection, reaching its peak 6 d post exposure. A viremic phase may be observed from days 2 till 8 after infection.

Most of the data available on the courses of the antibody responses were obtained by experimental respiratory infections in volunteers or with the mouse model. CV-neutralizing IgM antibodies, which may be serotype-specific or cross-reactive, appear 3 d after exposure (Figure 1). They reach their maximum titer level 1 wk after infection and typically disappear in the course of 3 mo[55-57,59,60].

Little is known on the anti-CV IgA responses. However, the sIgA and IgM responses seem to interfere with each other[60], which means the higher the one is the lower the other is with regard to their relative values. In some patients an IgA response is not detectable at all throughout the course of the infection. The presence of anti-CV IgA antibodies is detectable approximately 15 d post infection for the first time (Figure 1). These antibodies reach their peak level 21 d after exposure and disappear in the course of 6 wk[60].

The anti-CV IgG response approximately appears 4 d after exposure (Figure 1). These antibodies reach their maximum titer 2-3 wk after infection and may persist for years[55-57,61]. The humoral response plays a prominent role in limiting virus spread to different tissues by blood as well as in viral clearance[62]. In patients with agammaglobulinemia the infection spread to, and persisted in, the central nervous system[17,19,63]. CV infection of B cell-deficient mice results in chronic, high-titer infections in multiple organs, like heart, liver, lung and pancreas, and transfer of immune B cells at least transiently resulted in clearance of CV from all tissues[64]. Furthermore, the importance of antibodies is demonstrated by the finding that passive transfer of immune serum globulin reduces viral titers and symptoms in patients[65]. As demonstrated by passive immunizations with sera from fully recovered patients (within 72 h after infection), the presence of antibodies is also sufficient to prevent secondary infections.

In contrast to the evidently central protective role of anti-CV antibodies during coxsackievirus infections, some studies have shown that anti-CV IgG-mediated CV infection of monocytic/macrophagic cell lines, lymphocytes and plasmacytoid dendritic cells (pDC) *via* Fc receptors is possible[64,66-68], thus showing that IgG antibodies might contribute to virus dissemination in the body, enhance infection and exacerbate disease under certain circumstances. This effect must be taken into consideration in the development of vaccines.

The role of the T-cell responses in coxsackievirus infections is not clear. The data obtained with different mouse strains or different virus variants are controversial[21,62,69-74]. Using T cells directed against lymphocytic choriomeningitis virus-specific epitopes as sensors to evaluate antigen presentation by a recombinant CV expressing these epitopes, it was shown that the virus strongly inhibits antigen presentation through the MHC class I pathway[75], and therefore is able to evade $CD8^+$ T-cell immunity. In contrast to the low presentation by MHC class I molecules, MHC class Ⅱ-restricted presentation occurred at least at a level that might enable a primary CV-specific $CD4^+$ T-cell response[75].

Coxsackievirus infections are suspected to be involved in the induction of autoimmune reactions particularly against cardiac cells and pancreatic islet cells. However, a discussion of this aspect is beyond the scope of this review. In short, these reactions are directed against self-antigens by pre-existing auto-reactive lymphocytes. Coxsackieviruses might contribute to the activation of these lymphocytes by making more antigens available by the release of cellular components, which in addition might present novel, cryptic epitopes resulting from the cleavage of cellular proteins by the viral proteases 2A and 3C, during cytopathogenic infection and by promoting responses to these new antigens by presentation in an inflammatory context, which stimulates migration of lymphocytes to the relevant tissues[73]. There is no vaccine for active immunization against CV infections.

Human rhinovirus

After transmission by the respiratory route (sneezes) or

after infection by self-inoculation (hand into nose contact), the mean incubation time is 2 d. The typical symptoms are these of the common cold (rhinorrhea: running nose, swelling of the epithelial tissue), which last on average for 3 d. Virus shedding in the nasal secretions already occurs 8-10 h after exposure, reaches maximum levels between days 2-7 and sometimes may continue till day 14 after infection at very low levels[25,57,76]. Besides this common course of infection, virus might spread from the ciliated epithelial cells of the upper respiratory tract into the lower airways resulting in asthmatic exacerbations[77].

Anti-HRV SIgA in nasal secretions occur approximately 7 d after infection, a time point when the illness had subsided already, reaching their maximum level approximately 16 d post exposure[25] (Figure 1). In contrast to SIgA, sIgA to HRV does not increase before 6 wk after infection (Figure 1). The same applies for the anti-HRV IgG response, which develops between 6 and 7 wk after exposure and persists for approximately 1 year[25] (Figure 1). However, during secondary infections detectable amounts of serum antibodies to HRV may develop between 1 and 2 wk after infection, reaching their peak titer 5 wk post exposure[76,78].

The antibody response to HRV does not seem to play a role in virus spread and clearance, because it appears only after the end of the illness, and in persons with IgA deficiency and hypogammaglobulinemia normal recovery from illness occurs[79]. Furthermore, antibody production occurs on an average only in 50% of the cases and neutralizing antibodies, which do not show cross-reactivity, generally are produced in low amounts[76,78]. Therefore, protection by antibodies against secondary infections with HRV, which additionally appear in extraordinary different versions of serotypes, is strongly limited.

The T-cell response to HRV is incompletely understood. An increase in lymphocytes 3-4 d after exposure can be observed in nasal secretions. Specific $CD4^+$ T-cell clones, which secreted the Th-1 type cytokine IFN-γ, could be isolated in peripheral blood from persons with previous disease[80,81]. These T cells showed serotype cross-reactivity[80]. This implies that $CD4^+$ T cells can be activated by shared viral determinants, and can induce recall T-cell responses to HRV. One study suggests that eosinophils might act as antigen-presenting cells, which activate $CD4^+$ T cells[82]. No data are available on an involvement of cytotoxic $CD8^+$ T cells (CTL). There is no vaccine (neither passive nor active) against HRV infections.

HAV

After oral uptake, the mean incubation period is 4 wk. The preicteric period of normally 5 d with unspecific symptoms (nausea, malaise, headache) ceases with the onset of jaundice, which lasts on average for 3 wk. Fecal shedding of HAV already occurs during the late incubation period when no clinical symptoms are observable, and lasts for approximately 3 wk. The fecal excretion reaches its maximum just before the onset of hepatocellular injury and terminates about the time when the IgG antibody response is detectable (Figure 1). Viremia occurs a few days before and during the early acute stage and roughly parallels the fecal shedding, but at a lower magnitude. Besides this common course of infection, prolonged and relapsing courses occur in up to 25% of the patients[83].

Anti-HAV IgM antibodies are present in almost all patients at the onset of the symptoms (3-4 wk post infection). These antibodies reach their maximum level 2 mo post exposure, have only weak neutralizing activity and typically disappear in the course of 3 mo (Figure 1). But in the course of prolonged courses, IgM can be detected up to 1 year after onset of icterus[28,29,84,85].

Anti-HAV IgA antibodies are also detectable at the onset of the symptoms in blood (sIgA) (Figure 1). This response reaches its peak titer 50 d post exposure and may last for more than 5 years[28,29,85,86]. The majority of the IgA remains in circulation as sIgA and is not secreted into the intestinal tract as SIgA by the polymeric immunoglobulin receptor (pIgR) pathway. But a significant fraction of the sIgA may be released into the intestines *via* bile by liver functions mediated by the hepatocellular IgA-specific asialoglycoprotein receptor (ASGPR), and fecal samples contain IgA from 5 to 6 wk till 3 to 6 mo post infection[87-91] (Figure 1). Salivary anti-HAV IgA is also detectable in patients, which course parallels that of fecal IgA[90]. But the role of SIgA in the protection against HAV infections appears to be limited, as neutralizing activity in most human specimens is barely detectable, which correlates with animal studies[90]. Results obtained with cultured cells as well as in a mouse model suggest that HAV-specific IgA can serve as a carrier molecule for a liver-directed transport of HAV, supporting and enhancing the hepatotropic infection by uptake of HAV/IgA immunocomplexes *via* the ASGPR[91,92]. It could be shown that IgA-coated HAV is translocalized antivectorially from the apical to the basolateral site of cultured polarized epithelial cells *via* the pIgR[93], and it was assumed that fecal HAV/IgA[87,94], whose stability enables its fecal-oral transmission[91], is able to support the primary infection utilizing the IgA receptors. Furthermore, it was postulated that an enterohepatic cycling of HAV may be established during infection by HAV/IgA resulting in endogenous reinfections of the liver until large amounts of highly avid IgG displace the IgA in the HAV/IgA complexes[92]. Depending on the individual immune response, this mechanism may play a role in the development of the different courses of hepatitis A[92]. With respect to the anti-HAV IgA response in general, it is not clear by which processes and mechanisms induction occurs.

Neutralizing anti-HAV IgG antibodies are detectable 3-4 wk post infection for the first time, but this response develops slowly, reaching its peak titer 4 to 5 mo post infection (Figure 1), a time point late in the convalescence phase[28,29,85]. Anti-HAV IgG persists lifelong, although the titer may fall to undetectable levels after several decades. Although the minimum level of neutralizing antibodies that protects against infection and disease is unknown, an estimate of a minimal protective level is approximately

20 mIU/mL blood. Circulating anti-HAV IgG of the developing IgG response may limit viremia and thus re-infections at different hepatic sites by progeny virus, but is sufficient to prevent subsequent secondary infections. Passive immunization with pooled immune serum globulin[95,96] of at least 100 IU anti-HAV can prevent the disease for up to 5 mo with a certainty of 80%-90%. Definite duration of protection by immune serum globulin is dose related[97-100]. Studies suggest that passive immunization does not always prevent infection, but ensures an asymptomatic course of the disease[101,102]. IgG is still used for post exposure prophylaxis. If administered within 2 wk after exposure, either development of the disease is prevented or the severity of the disease is attenuated as well as virus shedding is reduced[95,96,103]. Since 1992, inactivated vaccines are available[104-106], which protect against both infection and disease caused by all strains of HAV with 100% efficacy for at last 10 years. Live, attenuated vaccines have been developed using virus adapted to growth in cell culture[107], but were poorly immunogenic[108]. Nonetheless, such a vaccine has been widely used in China and appears to be capable of inducing protective levels of antibody[109]. However, as anti-HAV IgA might be induced by live vaccines and act as pathogenicity factor for hepatitis A (see above), this approach might not be advantageous as compared to the inactivated vaccines.

Clearly, the antibody response to HAV prevents secondary infections and may limit viral spread during infection. But with regard to viral clearance and destruction of infected hepatocytes, anti-HAV antibodies do not seem to play a role. Destruction of infected hepatocytes by HAV-specific antibodies with or without the help of complement could not be demonstrated[29]. However, it has been shown that HAV-specific, HLA-restricted cytotoxic $CD8^+$ T lymphocytes (CTL) play a prominent role both in eliminating the virus and in causing liver injury (immunopathogenesis). CTLs were identified in liver biopsy specimens obtained during the acute infection[26,27]. Nearly 50% of the liver-infiltrating, cytotoxic T-cell clones displayed HAV-specific cytotoxicity. During activity the CTLs produced IFN-γ[27,110], which may stimulate HLA class I expression on hepatocytes and in the following promote upregulation of the normally low level display of antigen on liver cells resulting in more efficient destruction of infected cells by these CTLs. During this acute phase of infection only 1%-2% of the CTLs in peripheral blood showed HAV-specific cytotoxic activity[26], whereas 2-3 wk after onset of icterus[111], which means during the early convalescent phase, HAV-specific CTL activity reached peak levels in peripheral blood. This indicates that HAV-specific CTLs accumulate in the liver during the acute phase, and after the destruction of the infected hepatocytes leave the liver back into blood. Multiple dominant T-cell epitopes could be identified in the proteins VP1, VP2, VP3, 2B, 2C and mainly 3D[112]. This multitude of T-cell epitopes combined with an inhibitory effect of HAV on CTL-suppressing regulatory T cells during the acute phase of the disease[113] seems to result in a strong activity of HAV-specific CTLs leading to an efficient elimination of HAV, which might prevent persistence of the virus. Also NK T cells seem to be involved in the elimination of HAV and the destruction of hepatocytes[114].

INNATE IMMUNE RESPONSE

The innate immune system is designed to enable fast cell reactions to invading microorganisms. It is not aimed to respond to a specific pathogen, but pathogens are recognized by particular molecular patterns, which are specific for certain groups of pathogens (PAMP; pathogen-associated molecular pattern), but not found within cellular molecules. After recognition of viral molecular patterns, the synthesis of proteins, including a variety of cytokines and enzymes, and/or reactions, like apoptosis, are induced, which are able to interfere with the growth of the virus at the site of infection. Virus-specific molecular patterns are especially single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) (in the case of DNA viruses, unmethylated CpG DNA is additionally recognized by the TLR9). ssRNA represents viral RNA genomes either introduced by the invading virus or produced by virus replication, and dsRNA represents viral replication intermediates (in the case of dsRNA viruses it also represents the genome). As these viral nucleic acids can only be detected if they are freely present in the cytoplasm, sensing of viruses and induction of the cellular antiviral activities are only possible after the infection of cells has already occured. By far the most prominent reaction against viruses is the production and secretion of type I IFN by infected cells, resulting in the establishment of an antiviral state in the surrounding cells by expression of proteins with antiviral activity. Thus, viral spread in the infected tissue is contained. Type I IFN, which can be induced in virtually all cell types, represents numerous subtypes of IFN-α and a single IFN-β. The major cytoplasmatic recognition receptors are RIG-I[13,115,116] and MDA-5[12,13], which both are cytosolic and may associate with stress granules, as well as TLR3[117,118], which is localized in endosomal membranes. These three receptors activate the IKKε/TBK1 kinases [inhibitor of nuclear factor (NF)-κB kinase ε/TANK-binding kinase 1][119-122] *via* the adaptor proteins MAVS (mitochondrial antiviral signaling protein; RIG-I and MDA-5)[123-126] and TRIF (TIR domain-containing adaptor inducing IFN-β; TLR3)[127-129], respectively. These kinases phosphorylate interferon regulatory factor 3 (IRF-3), which results in IRF-3 dimerization and cytoplasmic-to-nuclear translocation[130,131], where it induces IFN-β transcription as a central component of the transcription complex[132,133]. Three additional transcription factors participating in the induction of IFN-β transcription are NF-κB, ATF-2 and c-Jun, which are activated by signaling pathways also starting from MAVS. After secretion, IFN-β binds to the type I IFN receptor (IFNAR1/2) on neighbouring cells, resulting in expression of a variety of interferon stimulated genes (ISGs) *via* the Jak/STAT signaling pathway, which additionally includes the transcription factor IRF-9 and the interferon

stimulated response element promoter element. In general, upon activation by viral stimuli these antiviral ISG proteins [e.g., protein kinase R (PKR) or oligoadenylate synthetase] affect the cellular, macromolecular synthesis (transcription, translation) and consequently viral growth. Induction of IFN-α requires the IFN-β-induced IRF-7, which is also activated by the IKKε/TBK1 kinases, but is not constitutively expressed as compared with IRF-3. Thereby, the effects of IFN-β are amplified in the course of the IFN response.

Besides induction of IFN synthesis in infected cells, apoptosis may be initiated by activation of caspases.

These antiviral effects are supported by cytokines, which are produced by monocytes/macrophages, dendritic cells, granulocytes (eosinophiles, basophiles and neutrophiles) and NK cells activated by cellular contents and debris released by the destruction of infected cells. For instance, cellular destruction may be caused by the cytolytic activity of cytopathogenic viruses. In this way, a hostile, inflammatory environment is created, which can be characterized by the presence and the amount of the different cytokines and of the different inflammatory mediators, which are induced by the cytokines in the cells at the site of infection (certain enzymes, prostaglandines/leukotriens, reactive oxygen intermediates). Simultaneously, the adaptive immune response is activated by effects of the cytokines (e.g., upregulation of antigen presentation).

In general, the effects caused by the innate immune system depend on the specific composition of the cytokine pattern, which is created by cell type-specific reactions of the cells involved in the antiviral response, including the leukocytes and the specialized cells of the tissue infected. Therefore, the innate immune response will vary according to the cell type infected. In addition, the response is influenced and modified in a specific manner by the ability of the viruses to interfere at certain sites of the cellular reactions (Figure 2). These specific interactions between host cell and virus significantly contribute to the pathogenesis of the infection, which can for example be clinically observed in the viral-specific course of the fever curve.

In the following, an overview of the interactions between PV, CV, HRV and HAV and the innate immune system is given.

PV

PV is partially resistant to type I IFN. In experiments using cells pre-treated with IFN-α, PV resistance against type I IFN correlated with the amount of virus infecting each cell[134], and it was shown that the viral protease 2A can inhibit the activity of ISG proteins. However, this ability depends on the cell type infected. In PV receptor-transgenic mice (only infectable because of this genetic modification), virus replication is limited to the central nervous system, whereas in mice which additionally lack the receptor for type I IFN, replication also occurs in liver, spleen and pancreas[135]. This presumably reflects cell type-specific antiviral effects of IFN which can not be inhibited by 2A. It is unknown which ISG proteins inhibit PV replication and by which mechanism 2A interferes with these proteins, but it was demonstrated that the IFN-inducible PKR is degraded by the PV protease 2A in cells infected with PV[136,137].

The release of IFN-β from cells infected with PV is repressed by a variety of mechanisms. Transcription, translation and secretion of IFN are affected by participation of the polioviral proteases 2A and 3C as well as by the protein 3A, which is able to interact with intracellular membranes. MDA-5 is degraded during infection in a proteasome- and caspase-dependent manner[138]. Although the mechanism involved is not fully understood, MDA-5 cleavage might be triggered by the proteases 2A and 3C, respectively (Figure 2). Both 2A and 3C mediate PV-induced apoptosis[139,140], causing mitochondrial damage, release of cytochrome c, and activation of the caspases 3 and 9[141]. The caspases might produce cleavage products of MDA-5, which are substrates for the proteasome. The apparent disadvantage of inducing apoptosis in cells infected can be compensated by the fast replication of PV, and the apoptosis-induced MDA-5 cleavage resulting in suppression of IFN synthesis provides the opportunity that newly synthesized viruses are able to infect neighbouring cells, in which no antiviral status is established. However, inhibition of MDA-5 cleavage did not influence PV replication[138], indicating that alternative signaling pathways for IFN induction are available after PV infection. In this context it is remarkable that PV-3C cleaves RIG-I[142]. This might indicate that this sensor of viral nucleic acids, too, is involved in the recognition of picornaviral RNA, although it is believed that MDA-5 is the major sensor receptor. Not only are both cytoplasmatic RNA sensors cleaved during PV infections, but also is the mitochondrial protein MAVS that is transmitting the signal from the sensors downstream cleaved by the proteases 2A and 3C, in which 3C seems to be positioned at the mitochondrial membrane by 3A of the processing intermediate 3ABC[143]. Therefore, different sites inside the RIG-I/MDA-5 pathway are attacked by PV, which might result in cooperative or synergistic effects, and might compensate for the only partial resistance of PV to IFN.

The viral proteases are not only involved in the inactivation of components of the signaling pathway resulting in induction of IFN transcription, but also cleave other cellular proteins, including eIF4G[144] (cleaved by 2A), which is necessary for cap-dependent initiation of cellular protein translation, and PABP[145] (cleaved by 3C), resulting in an attenuation of IFN-β translation (Figure 2).

Besides inactivation of cellular proteins necessary for expression of IFN-β by proteolytic cleavage through viral proteases, a significant reduction in secretion of IFN-β as well as of the pro-inflammatory cytokine interleukin-6 (IL-6), is caused by localization of the viral 3A protein to the ER leading to an attenuation of the ER-to-Golgi traffic[146] (Figure 2). This mechanism results in a diminished IFN response as well as in an attenuation of

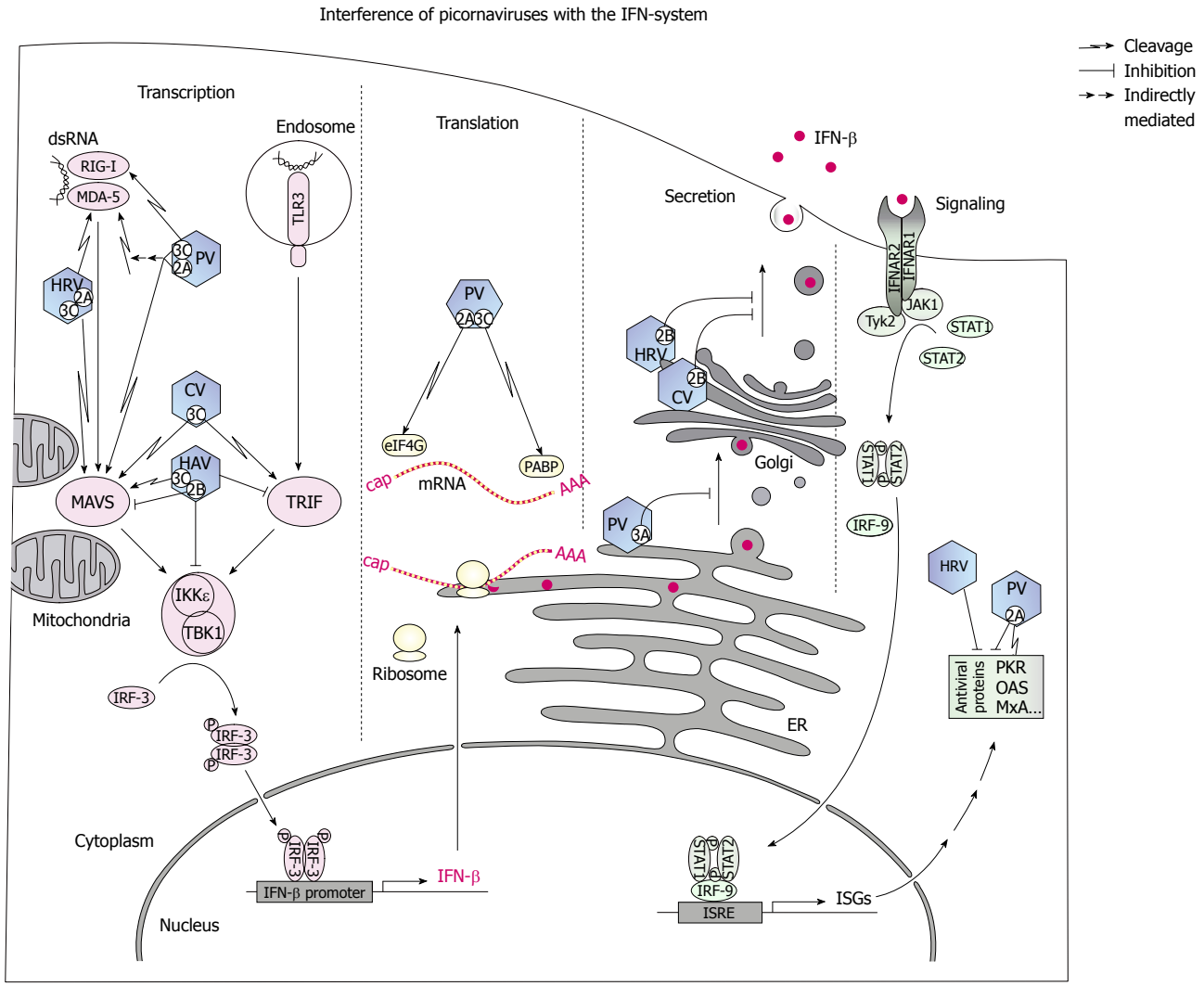

Figure 2 Interference of picornaviruses with the interferon system. This figure schematically shows the sites and effects of the interference of poliovirus, coxsackievirus, human rhinovirus and hepatitis A virus with the interferon-β response. For more details we refer to the text. PV: Poliovirus; CV: Coxsackievirus; HRV: Human rhinovirus; HAV: Hepatitis A virus; IFN: Interferon; IRF: Interferon regulatory factor; ISGs: Interferon stimulated genes; MDA: Melanoma differentiation-associated gene; dsRNA: Double-stranded RNA; PKR: Protein kinase R; TLR: Toll-like receptor; MAVS: Mitochondrial antiviral signaling protein; ISRE: Interferon stimulated response element; OAS: Oligoadenylate synthetase; RIG-I: Retinoic acid-inducible gene I.

inflammation. Additionally, the rate of MHC-I transport to the cell surface is reduced, resulting in an inhibition of antigen presentation and therefore of the adaptive $CD8^+$ T-cell response.

In summary, PV seems only to be able to interfere partially with certain sites of the signaling and synthetic pathways participating in the innate immune response to the virus. But the virus seems to be capable to dampen the innate responses to a certain degree by interference with multiple sites in these pathways, allowing the virus to establish infection.

Coxsackie virus

The innate immune response against CV was investigated in the presence of different post-acute symptoms, utilizing different cell types in cell culture experiments as well as different mouse strains and different virus strains, and accordingly, the available data is versatile. We will give a summary on the processes found that apply in general. CV is sensitive to IFN-β, and treatment of patients with myocardial virus persistence with this cytokine results in elimination of the virus[147]. The protective role of the IFN-β system was demonstrated in mice lacking the type I IFN receptor and in IFN-$\beta^{-/-}$ mice, respectively[148,149]. In these mice the susceptibility to infection as well as the severity of the disease was significantly increased. These findings correlate with results obtained with mice deficient in the genes for MDA-5[150,151] and TLR3[152], respectively. The pronounced effect on mortality in MDA-5/TLR3 double-knockout mice after CV infection might indicate a cooperative role of these receptors[151]. These results show that MDA-5 and TLR3 are involved in IFN-β induction during CV infections. However, this does not influence virus titers[151]. It is possible that type I IFN reduces mortality during infection independent of its effect on viral replication. Nevertheless, CV is able to attenuate the IFN response by protease 3C-mediated cleavage of the adaptor protein MAVS, which

transmits the signal from MDA-5 to the IRF-3 kinases, and the adaptor protein TRIF, which transmits the signal from TLR3 to the IRF-3 kinases[153] (Figure 2). Cleavage of these adaptors also inhibits apoptotic signaling. In addition, secretion of IFN and other cytokines, which are induced by other pathways, as well as MHC-I transport to the cell surface for antigen presentation, are inhibited by the viral protein 2B, which localizes to the Golgi complex, thus inhibiting trafficking through the Golgi[154-157] (Figure 2). The intercalation of CV-2B into the Golgi membranes also results in down-regulation of Ca^{2+} signaling between Golgi and mitochondria and consequently in suppression of the apoptotic cell response[158].

Besides TLR3, coxsackievirus infections can be also sensed by further TLRs[62], including TLR7 and TLR8[67,159], which recognize viral ssRNA. These receptors can mediate production of pro-inflammatory cytokines and type I IFNs in human cardiac cells and pDC. But this response seems to occur late in the course of the infection, after the antibody response to CV has developed, as TLR7 activation seems to appear after the IgG-mediated entry of CV into pDCs *via* the Fc receptor, and absence of MyD88, the adaptor molecule for TLR7, does not affect the mortality rate of CV infections[151]. Amazingly, TLR4, which is normally activated by bacterial lipopolysaccharides, appears to be activated by CV resulting in secretion of pro-inflammatory cytokines, which correlates with a more severe course of the disease[160-163]. However, the mechanism of activation by CV is unknown. Although CV can be recognized by several receptors sensing viral nucleic acids, TLR3 seems to play a critical, non-redundant role. TLR3 deficient mice are unable to control CV replication, and activation of alternative pathways is not sufficient to protect the host[152,164].

It has been demonstrated that several innate effector cell types, including NK cells, macrophages and dendritic cells are involved in the secretion of the cytokines and pro-inflammatory mediators during the innate response to CV[73]. Depletion of NK cells, which are involved in the maturation of DCs and activation of T cells, substantially increased CV titers in the heart or pancreas[165]. Concerning the role of macrophages, it has been demonstrated that inducible nitric oxid synthase expressing macrophages migrate to CV-infected tissues, that inhibition of this enzyme results in higher viral titers[166], and that adoptive transfer of macrophages from wild type mice has protective effects in TLR3-deficient mice by reducing cardiac disease and mortality following CV infection[152]. With regard to dendritic cells, their subset composition and functionality have an impact on the development of myocarditis[167].

The role of CV binding to the complement component C3 and activation of the alternative pathway[168] is not clear, but might be involved in the development of myocarditis by the effects of C3a and C5a on activation of leukocytes.

A whole series of different substances (cytokines, chemokines and mediators of inflammatory reactions) and cell types of the innate immune system show correlations with autoreactive processes following CV infection. However, it has not clearly been shown in which way they contribute to the disease. But the balance of timing, duration and amount of expression of the different cytokines, which depends on the strength of signaling from the different pathways and results in a certain cytokine pattern, seems to be important and critical for the development of an appropriate antiviral response that does not degenerate into autoimmunity (for review see Richer 2009[152]).

Human rhinovirus

During HRV infection, a variety of antiviral factors are released by the epithelial cells including the vasoactive peptide bradykinin and the pro-inflammatory cytokines IL-1β, tumor necrosis factor α, IL-6 and IL-8, which activate granulocytes, dendritic cells and monocytes to migrate to the site of infection[169,170]. IL-8 especially attracts neutrophiles, which might contribute to the exacerbation of asthma observed in infections[171]. Type I IFN, to which HRV is sensitive[172], is detectable in nasal secretions in approximately 30% of the patients[78], but up-regulation of the IFN-induced MxA protein in the nasal mucosa could not be detected[173]. As HRV can replicate in certain cells pre-treated with IFN-α[134], the virus has the ability to interfere with the activity of ISG proteins and attenuate the effects of type I IFN. However, the mechanisms involved are unknown.

Although HRV seems to be eliminated by the innate immune responses, the virus has the ability to affect several components of the IFN-β induction pathway (Figure 2). At least certain types of HRV are able to cleave the cytosolic viral pattern recognition receptors MDA-5 (type 1a)[138] and RIG-I (type 16)[142], respectively. Cleavage of the mitochondrial protein MAVS also could be demonstrated during HRV 1a infection[143] (Figure 2). This cleavage is mediated by the viral proteases 2A and 3C. Here, the activity of 3C seems only to be displayed by the processing intermediate 3ABC, which is directed to the mitochondrial membrane by the function of 3A. However, 2A as well as 3C mediate the HRV-induced apoptosis[174], which has antiviral effects. But caspase-3, which is activated during apoptosis, is able to support the 2A/3C-mediated inhibition of MAVS signaling by cleavage of MAVS[143]. Furthermore, the secretion of cytokines, including IFN, is inhibited by HRV-2B, which localizes to the Golgi membranes resulting in the inhibition of the secretory pathway[157]. This effect might also delay MHC-I presentation. By this attenuation of the IFN-β response, the fast replicating HRV might gain time to establish infection at least locally and thus to secure its transmission.

HRV is also able to attenuate the inflammatory effects caused by the cytokines released from epithelial cells during infection by induction of IL-10 secretion in monocytes and macrophages[76,175]. The mechanism by which HRV causes this effect is unknown. IL-10 does not only inhibit the production of pro-inflammatory cytokines[176],

but also downregulates MHC class Ⅱ molecule expression[177], which results in inhibition of T cell activation. This inhibitory effect on T-cell stimulation might contribute to the delayed adaptive immune response against HRV and might be supported by additional viral effects on antigen presenting cells[76]. Although HRV does not infect leukocytes, the virus binds to these cells *via* the intercellular adhesion molecule-1[178], which serves as cellular receptor of the major group of HRV, and is important in leukocyte migration and stimulation of T-cell responses. This binding results in an enhanced adhesiveness of monocytes/macrophages[179], which thereby might be retained longer at the infected sites. As a result, delayed emigration of these cells to lymph nodes might occur, which in combination with an attenuated stimulatory effect on T-cell proliferation might inhibit T-cell activation. An additional inhibitory effect on the adaptive T-cell response might be due to HRV-induced upregulation of CD274 and sialoadhesin (Siglec-1) on dendritic cells[180]. Both molecules inhibit the T-cell stimulatory function of DCs, but the mechanisms involved are unknown.

HAV

Experimental elimination of HAV infections in human fibroblast cultures by exogenously added IFN-α/β showed that HAV is not resistant to these IFNs[9,181] but reports on the presence of type I IFN during the acute phase of HAV infections are controversial. Some indicate that patients do not produce IFN[182-184], while in other reports evidence for the presence of IFN is announced[185-187].

At the cellular level, HAV infections result in a persistent noncytopathic infection[9,188,189] and neither measurable IFN-α/β levels[9,190] nor interference with the infection by other viruses[190] could be detected in lymphocytes and fibroblasts infected with HAV. Further investigations showed that HAV does inhibit IFN-β transcription[191] by effectively blocking IRF-3 activation[192], presumably due to a cooperative effect of the HAV proteins 2B and 3ABC[193] (Figure 2). While MAVS is targeted for HAV protease 3C-mediated proteolysis by 3ABC, an intermediate product of HAV polyprotein processing localized to mitochondria by 3A[194], 2B seems to interfere with MAVS as well as with the kinases IKKε/TBK1 by a so far unknown mechanism[193]. It is assumed that the effects of 2B on MAVS and the kinases indirectly result from interactions of 2B with cellular membrane structures. It could also be demonstrated that HAV is able to affect the TLR3 transduction pathway by direct interaction with TRIF[192] (Figure 2).

These results strongly suggest that IFN-β does not play a role in preventing HAV infections, and that the ability of HAV to interfere with the RIG-I/MDA-5 signaling pathway allows this slowly replicating virus to establish infection. Furthermore, this strategy of inhibiting IRF-3 activation through interference with MAVS and the kinases may allow HAV to preserve the infection for a longer time by preventing IRF-3-mediated down-regulation of the liver cell metabolism[195], and by evading the cellular IFN response at later stages of infection, a time point when RIG-I/MDA-5 may be upregulated by IFN-γ secreted by HAV-specific CTLs (see "Adaptive Immune Response"), enhancing cell responsiveness to viral RNA[196,197].

HAV also has the ability to prevent apoptosis induced by accumulating dsRNA[191], but the underlying mechanism is not clear. It was found that HAV enhances activation of the transcription factor NF-κB[192], and as this pleiotropic factor is involved in expression of anti-apoptotic genes[198], the ability of HAV to activate NF-κB might play a role in the inhibition of apoptosis.

A transient suppression of hematopoiesis with granulocytopenia is frequently observed in the preicteric phase of HAV infections[199,200], and in studies with long-term human bone marrow cultures, HAV-induced inhibition of hematopoiesis was demonstrated[201-203]. As shown with human peripheral blood monocytes, inhibition of the differentiation of monocytes to macrophages by HAV may be involved in the perturbations of hematopoiesis[204]. This might result in an attenuation and retardation of the inflammatory response and of the induction of the adaptive immune response against HAV. The mechanism of this effect is not known. The importance of NK cells for the elimination of HAV is controversially discussed[110,205,206].

CONCLUSION

The data presented here give an overview of the immune responses against PV, coxsackievirus, human rhinovirus and HAV, which are the four best-studied members of the picornavirus family. They illustrate that much is known about the defense mechanisms of the human host against infection with these viruses and about the viral countermeasures, but also that many open questions exist. The immune responses against these viruses, like against other viruses, are complex and as diverse as the viruses themselves.

Processes which are demonstrated for a particular virus do not necessarily also apply to an other virus of this family, not even to a different strain of the same virus. In many cases the viruses were not examined under the same conditions and circumstances. But there may also be similarities between the different viruses which have not been investigated or clearly shown so far. It becomes evident that the time points at which certain responses occur during an infection are very variable and seem to depend on the tissue/cell type infected as well as on individual physiological conditions of the patient, like age or immune status.

Although investigated for many years, some data are incomplete, like type and duration of the antibody responses. The significance of some findings is not clear, as they were obtained using animal models or cultivated cell lines, which do not represent the natural targets for the viruses. For some observations and findings, the mechanisms involved are unknown. For example, it is not totally clear which intracellular receptors are involved in the sensing of picornaviral infections, which signaling path-

ways are involved in cytokine production or mediate the effects of the cytokines, and which signaling pathways are involved in the induction of the expression of cellular receptors participating in the regulation of the immune responses. It is not clear which role the innate immunity plays in recovery from acute infection, and whether the viruses are able to persist, at least for a certain time, in patients with immunoglobulin deficiencies as well as in immunocompetent hosts, and if, in which cell type.

It is not only of importance to find out by which abilities and mechanisms the human picornaviruses interfere with the immune system, but also to identify certain pathogenicity factors which allow the viruses to establish and maintain infection, like the picornaviral 3C protease, which inhibits induction of IFN-β by cleavage of RIG-I/MDA-5 signaling components. This knowledge may allow the prediction of interspecies transmissions from animals to humans by certain members of this large, heterogeneous family. The risk of such transmissions resulting in new emerging diseases is evident by recent epidemics caused by viruses like SARS or hemorrhagic fevers.

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Events Calendar 2012

January 13-14, 2012
Innovation in Severe Acute Respiratory Infections
Sitges, Spain

January 20, 2012
Exploiting Bacteriophages for Bioscience, Biotechnology and Medicine
Welwyn Garden City,
United Kingdom

January 20-22, 2012
International Science Symposium on HIV and Infectious Diseases
Chennai, India

February 26-29, 2012
10th American Society for Microbiology Biodefense and Emerging Diseases Research Meeting
Washington, DC, United States

March 11-15, 2012
8th International Symposium on Pneumococci and Pneumococcal Diseases
Foz de Iguaçu City, Brazil

March 12-16, 2012
Vaccine World Summit India
Hyderabad, India

March 14-17, 2012
22nd Annual Meeting of the German Society for Virology
Essen, Germany

March 21-26, 2012
2012 HIV Vaccines
Keystone, CO, United States

March 21-26, 2012
Viral Immunity and Host Gene Influence
Keystone, CO, United States

March 26-31, 2012
Cell Biology of Virus Entry, Replication and Pathogenesis
Whistler, BC, Canada

March 26-31, 2012
Frontiers in HIV Pathogenesis, Therapy and Eradication
Whistler, BC, Canada

April 20-21, 2012
2012 Molecular Virology Workshop
Daytona Beach, FL, United States

April 22-25, 2012
2012 28th Clinical Virology Symposium
Daytona Beach, FL, United States

April 24-27, 2012
European Molecular Biology Organization Workshop - Antigen presentation and processing
Amsterdam, Netherlands

May 19-22, 2012
European Molecular Biology Organization and European Molecular Biology Laboratory Symposium - New perspectives on immunity to infection
Heidelberg, Germany

June 11-16, 2012
Antiviral RNAi: From Molecular Biology Towards Applications
Pultusk, Poland

June 28-29, 2012
7th International Workshop on Hepatitis C - Resistance and New Compounds
Cambridge, MA, United States

July 16-20, 2012
European Molecular Biology Organization Conference Series - Viruses of microbes: From exploration to applications in the -omics era
Brussels, Belgium

July 19–20, 2012
7th International Workshop on HIV Transmission - Principles of Intervention
Washington, DC, United States

July 21-25, 2012
31st Annual Meeting of American Society for Virology
Madison, WI, United States

July 30 - August 1, 2012
3rd Annual Symposia of Hepatitis Virus
Guangzhou, China

August 20-22, 2012
2nd World Congress on Virology
Las Vegas, NV, United States

September 7-9, 2012
Viral Hepatitis Congress 2012
The Johann Wolfgang Goethe University, Frankfurt, Germany

October 18-20, 2012
2nd World Congress on Controversies in the Management of Viral Hepatitis (C-Hep)
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November 28 -December 1, 2012
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Format

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1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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