Contributions of the lymphocytic choriomeningitis virus glycoprotein and polymerase to strain-specific differences in murine liver pathogenicity

Andreas Bergthaler, Doron Merkler, Edit Horvath, Lukas Bestmann and Daniel D. Pinschewer

Correspondence
Andreas Bergthaler
andi@pathol.unizh.ch

1Institute of Experimental Immunology, Department of Pathology, University Hospital of Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland
2Department of Neuropathology, Georg August University, Robert-Koch-Strasse 40, 37075 Göttingen, Germany
3Institute of Clinical Chemistry, University Hospital of Zurich, Ramistrasse 100, 8091 Zurich, Switzerland

Received 28 July 2006
Accepted 29 September 2006

Hepatic involvement is commonly observed in arenavirus infections, but the viral determinants of liver disease are only partially understood. Here we exploited newly developed reverse-genetic techniques with Lymphocytic choriomeningitis virus (LCMV), the prototype arenavirus, to address specifically the contribution of the viral glycoprotein (GP) to liver pathogenicity. It is well established that strain WE, but not ARM, causes hepatitis in mice. We found that this property correlated with the superior capacity of WE to propagate in cultured macrophages and hepatocyte-derived cells. In mice, the ability to establish prolonged viraemia allowed the virus to propagate from initially infected Kupffer cells in the liver to neighbouring hepatocytes that underwent apoptosis. Reverse-genetic replacement of the GP in strain ARM with WE-GP resulted in only a very modest increase in liver pathogenicity, if any. Yet, an ARM-derived variant virus with a mutated polymerase gene caused severe liver disease when engineered to display WE-GP but considerably less when expressing ARM-GP. This reverse-genetic approach to an animal model of arenaviral hepatitis reveals a previously underestimated contributory role of the GP that alone is, however, insufficient to cause disease.

INTRODUCTION

Arenaviruses are distributed in a variety of rodent species worldwide and they are usually apathogenic in their natural hosts (Buchmeier et al., 2001). However, accidental transmission to humans can lead to severe disease, commonly with substantial involvement of the liver. Monkeys in captivity develop 'callitrichid hepatitis' when infected with the prototypic arenavirus Lymphocytic choriomeningitis virus (LCMV) (Montali et al., 1995). Similarly, LCMV infection of human adults can cause liver abnormalities, including transaminase elevation and hepatocellular necrosis (Fischer et al., 2006), and, in a recent outbreak of the New World arenavirus Whitewater Arroyo virus, high lethality was attributed to liver failure (Centers for Disease Control and Prevention, 2000). Notably, serum transaminase activity, a biochemical parameter of viral hepatitis, in addition to viraemia, represents the primary predictor for survival in human Lassa fever (Johnson et al., 1987).

When viscerotropic isolates are administered systemically and at high doses to adult mice, the natural host of LCMV, severe hepatitis ensues, manifest in vastly elevated serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities (Leist et al., 1989; Zinkernagel et al., 1986). LCMV has therefore also been referred to as 'lymphocytic choriomeningitis virus' (Hotchin, 1962). Pathogenesis in this model is largely attributed to immunopathological mechanisms (Balkow et al., 2001; Leist et al., 1989), with the severity of disease depending on various parameters of virus–host balance, namely the virus strain, infectious dose, route of infection and MHC haplotype (Leist et al., 1989; Zinkernagel et al., 1986). Accordingly, T-cell dependence is a hallmark of this disease in mice (Zinkernagel et al., 1986), whereas hepatocellular dysfunction in LCMV-infected monkeys has recently been correlated with the capacity of the virus to interfere with the proline-rich homeodomain protein PRX/HEX (Djavani et al., 2005).

Arenaviruses have a single-stranded negative-sense RNA genome consisting of a small ~3.4 kb (S) and a large...
Moreover, the two strains carry the same H-2b-restricted approximately 84% sequence identity at the nucleotide level. Among the most widely studied LCMV isolates are the (Pinschewer in transcription termination and particle formation region (IGR) located between the ORFs plays a dual role promoters (Perez & de la Torre, 2003) and the intergenic region (IGR) located between the ORFs plays a dual role in transcription termination and particle formation (Pinschewer et al., 2005).

Among the most widely studied LCMV isolates are the Armstrong (ARM) and WE strains. Their genomes exhibit approximately 84% sequence identity at the nucleotide level and they share 91% amino acid sequence identity. Moreover, the two strains carry the same H-2k-restricted CTL epitopes. ARM is widely considered to be neurotropic (Armstrong & Lillie, 1934), whereas the WE strain is generally described as viscerotropic (Rivers & Scott, 1935). Accordingly, it is well documented that WE but not ARM causes liver disease in mice and also systemic haemorrhagic manifestations with severe liver involvement in monkeys and guinea pigs (Lukashevich et al., 2002, 2004; Peters et al., 1987; Riviere et al., 1985; Zinkernagel et al., 1986).

Reassortants between the S and the L segment of ARM and WE have been generated (Kirk et al., 1980; Riviere et al., 1985; Romanowski & Bishop, 1983). Using these reassortants, both the haemorrhagic disease in guinea pigs and the functional disturbance of hepatocellular homeostasis in culture have been mapped to the L segment of WE (Djavani et al., 2005; Riviere et al., 1985), whereas to our knowledge the genetic basis of liver disease in mice has not yet been investigated. In guinea pigs, it had been noted that the ARM(L)/WE(S) reassortant was somewhat more virulent than ARM, whereas WE(L)/ARM(S) was slightly attenuated compared with WE (Riviere et al., 1985), indicating a contributory role of the S segment. The genetic basis thereof, i.e. the contribution of individual genes (e.g. GP vs NP on the S segment) and of non-coding cis-acting regulatory RNA sequences (5'UTR, 3'UTR and IGR), could not be differentiated by the reassortant technique, however. Considering the profound effects of GP on the viral phenotype in various LCMV disease models (Sevilla et al., 2000; Teng et al., 1996), we hypothesized that GP might also contribute to the ability of the virus to cause hepatitis in mice. However, based on the guinea pig and monkey studies, a key role was also expected for L segment-encoded determinants. Using recently developed recombinant cDNA technology to modify the S segment of LCMV, we demonstrate here a previously unknown and unexpected role of the LCMV GP in liver disease of mice.

**METHODS**

**Mice and animal experiments.** C57BL/6 and interferon type I receptor-deficient RAG2 double-deficient mice [A−/− R−/− mice (Grob et al., 1999), used exclusively for the isolation of clone 6] were bred at the Institut für Labortierkunde, University of Zurich. Animal experiments were performed under specific-pathogen-free conditions with authorization of the Veterinäramt of the Kanton Zürich and in accordance with Swiss law for animal protection.

**Cell lines and viruses.** Cell lines MC57G (ATCC CRL-2295), BHK-21 (ATCC CCL-10) and TIB-75 (ATCC TIB-75) were originally provided by M. J. Buchmeier (The Scripps Research Institute, La Jolla, CA, USA) and by F. Lehmann-Grube (Heinrich-Pette Institut, Hamburg, Germany), respectively. The generation of rARM/INDG (Pinschewer et al., 2003b) and rARM/ARMGP (previously referred to as rLCMV-ARM; Berghaler et al., 2006) has been described previously. rARM/WEGP, rCL6/WEGP and rCL6/ARMGP have been generated in this study. Clone 6 was isolated from an A−/− R−/− mouse persistently infected with rARM/INDG for > 200 days (A. Berghaler and D. D. Pinschewer, unpublished) and was plaque purified. Virus stocks were grown on L929 cells (wtWE) or BHK-21 cells (remaining viruses) and were titrated by immunofocus assay on MC57G cells (Battegay et al., 1991).

**RT-PCR and sequence analysis.** Processing of total cellular RNA for detection of INDG and LCMV-ARM NP RNA by RT-PCR has been described previously (Pinschewer et al., 2003b). For detection of WE-GP RNA, primers 5'-CCTTCACTAAGGACTCATCC-3' and 5'-GAACTTGTGAATTAAGATAGTGG-3' amplified a 794 nt product. PCR conditions are available from the authors upon request. The clone 6 L segment was sequenced as described by Flatz et al. (2006), covering all coding and non-coding elements except for the terminal 26 and 25 nucleotides of the 5' and 3' UTRs, respectively (primer binding sites).

**Serum transaminases.** Mouse serum was prediluted 1:4 in PBS and AST and ALT activities were determined on a Roche/Hitachi Modular Analytics system according to the recommendations of the International Federation of Clinical Chemistry with pyridoxal phosphate activation at 37°C.

**Cytotoxicity assays and enumeration of epitope-specific CD8+ T cells.** Specific CTL activity of splenocytes was assayed in a 51Cr release assay (spontaneous release always <17%) and epitope-specific CD8+ T cells were enumerated using MHC class I tetramers as described by Berghaler et al. (2006).

**Plasmids.** pC-L and pC-NP have been described previously (Pinschewer et al., 2003a). For generation of Pol-I-S_WEGP(−) (for intracellular expression of the rARM/WEGP S segment), the WE-GP ORF (GenBank accession no. AJ297484; Beyer et al., 2001) was amplified by PCR for insertion into the pSpam(−) backbone (Pinschewer et al., 2003b) by a cloning strategy outlined previously in detail (Pinschewer et al., 2003b). An analogous poll-driven vector for intracellular expression of a wtARM S segment [Pol I-S_ARMGP(−)] has been described previously (GenBank accession no. DQ458914; Berghaler et al., 2006).

**Immunohistochemistry and immunofluorescence.** After transcardial perfusion with saline followed by 4% paraformaldehyde, liver tissue was post-fixed overnight in fresh fixative. Paraffin-embedded 3 μm-thick sections were stained with haematoxylin-eosin (H&E). Immunohistochemistry was performed after unmasking of
antigen by microwave treatment (15 min, 800 W) in citrate buffer and blocking (10% FCS in PBS for 10 min at room temperature). LCMV antigen was detected using rabbit serum (Gossmann et al., 1991). CD8+ T cells were stained using rat anti-mouse CD8 (BD PharMingen). Liver tissue was prepared in HOPE fixative (DCS Innovative; Oler et al., 2001). Bound antibody was visualized using an avidin–biotin technique with 3,3′-diaminobenzidine as chromogen.

Immunofluorescence double-staining was performed on snap-frozen, acetone-fixed liver sections, blocked with 10% FCS and goat anti-mouse Fab (Jackson ImmunoResearch). Hepatocyte paraffin-1 antibody (HepPar1; NeoMarkers) was combined with LCMV NP-specific mAb VL-4 (Battetay et al., 1993). Alternatively, rat anti-mouse F4/80 (Serotec) was combined with rabbit anti-LCMV serum (Gossmann et al., 1991). Bound antibody was visualized with Cy3- or Cy2-conjugated goat anti-rabbit IgG, donkey anti-rat IgG or donkey anti-mouse IgG (all from Jackson ImmunoResearch). TUNEL staining was performed with a Roche Applied Systems kit.

Quantitative analysis of CD8+ T-cell infiltrates, apoptotic hepatocytes and cell type-specific virus distribution in the liver. Liver infiltrating CD8+ T lymphocytes were enumerated as described previously (Maini et al., 2000). Briefly, using an ocular morphometric grid at 400× magnification, intralobular and perilobular CD8+ T cells were counted in 10 high-power fields (HPF) to calculate the number of cells per HPF.

For enumeration of apoptotic hepatocytes, overview photographs (40×) of liver sections were measured and apoptotic hepatocytes (identified at 400× magnification by their highly condensed TUNEL-positive chromatin masses) were enumerated.

For counting of LCMV antigen-positive cells co-expressing HepPar1 (hepatocytes) or F4/80 (Kupffer cells), random areas of at least 9.1×103 μm2 were acquired on a fluorescence microscope (Olympus Optical).

All analyses were done with Analysis Software Colour View II Soft Imaging System (Germany).

Statistical analysis. For the assessment of between-group differences of multiple groups, we performed one-way or two-way ANOVA with Bonferroni’s multiple comparisons. Single measurements of two groups were compared with two-tailed Student’s t-tests. Graphpad Prism software (version 4.0b) or SPSS version 12.0 were used. We considered P values < 0.01 as highly significant (indicated as **), P values < 0.05 as statistically significant (indicated as *) and P values > 0.05 as not significant (indicated as n.s.).

RESULTS

Generation of a recombinant ARM-based LCMV expressing the GP of WE (rARM/WEGP)

To study the role of the viral glycoprotein in WE-induced liver disease, we relied on a partial rescue system that uses an artificial helper virus as a source for the L segment (depicted schematically in Fig. 1a; see also Bergthaler et al., 2006). A wtARM-based virus expressing the GP of the WE strain (Fig. 1b) was generated by transfecting BHK-21 cells with a polI-driven vector expressing intracellularly an engineered ARM-based S segment encoding a WE-GP gene (identical to the master consensus sequence of the wtWE virus used in this study, GenBank accession no. AJ297484) in place of the master consensus sequence of the wtWE virus used in this study, GenBank accession no. AJ297484) in place of

![Image](https://example.com/image1.png)

**Fig. 1.** Genome organization, generation and characterization of the rARM/WEGP recombinant virus. (a) Description of the recovery protocol that yielded rARM/WEGP. ‘lim. dilution/ plaque pur.’ Limiting dilution followed by plaque purification. (b) Schematic of the wtARM, wtWE, rARM/ARMGP and rARM/WEGP genomes. Arrows indicate ORFs. Inverted writing (NP, L) indicates antisense polarity. wtARM and wtWE are wild-type WE-GP genomes. Arrows indicate ORFs. Inverted writing (NP, L) indicates antisense polarity. wtARM and wtWE are wild-type virus isolates, whereas rARM/ARMGP and rARM/WEGP carry cDNA-derived S segments and a helper virus-derived ARM L segment. The rARM/WEGP S segment consists of an ARM-NP gene and ARM-derived non-coding sequences combined with a WE-GP gene. rARM/ARMGP is of wtARM sequence (Bergthaler et al., 2006). Genetic elements are hatched (WE) or white (ARM) to indicate their origin. (c) Viral genotype analysis by RT-PCR. Arrows indicate gene-specific amplification products. All amplification products were RT-dependent (‘−RT’), excluding residual plasmid contamination as PCR template.
ARM-GP. Cotransfection of polII-driven plasmids expressing the minimal viral transacting factors NP and L resulted in the intracellular reconstitution of an engineered S segment ribonucleoprotein. Subsequent infection with an engineered LCM helper virus expressing the vesicular stomatitis virus GP instead of LCMV GP (rARM/INDG, also referred to as rLCMV/INDG; Bergthaler et al., 2006; Merkler et al., 2006; Pinschewer et al., 2003b, 2004) provided an ARM-derived L segment for reassortment. The viral progeny therefore consisted of rARM/INDG helper virus mixed with a fraction of rARM/WEGP. Passage of this viral population in the presence of INDG-specific neutralizing antibody eliminated the rARM/INDG helper virus, and rARM/WEGP free of detectable helper virus contamination (≤1 p.f.u. in 10^4 p.f.u. rARM/WEGP as determined by RT-PCR; Fig. 1c) was isolated by plaque purification.

Genetic fingerprint analysis of rARM/WEGP by RT-PCR confirmed that the virus expressed WE-GP in combination with ARM-NP as expected, and immunofocus reduction assays were carried out to verify its seroreactivity (see Supplementary Table S1 in JGV Online). rARM/WEGP was neutralized by anti-LCMV-WE but not by anti-VSV-INDG neutralizing antibodies (that neutralized the rARM/INDG helper virus). Moreover, rARM/WEGP was neutralized by the WE-GP-specific monoclonal antibody WEN1 (Seiler et al., 1998), whereas wtARM or a genetically engineered ARM-based virus carrying a cDNA-derived wtARM S segment were not rARM/ARMGP, previously referred to as rLCMV-ARM* (Bergthaler et al., 2006), Fig. 1b, GenBank accession no. DQ458914]. The GP of rARM/ARMGP is identical to the master consensus sequence of the wtARM virus used in this study (Grande-Perez et al., 2005; GenBank accession no. AY847350). We have previously reported that rARM/ARMGP and wtARM reached identical titres in infected mice and were cleared with the same kinetics (Bergthaler et al., 2006). Moreover, the two viruses caused equally mild liver disease, as reflected in serum AST and ALT activity (Supplementary Fig. S1), and hence they were used throughout this study as indicated in text and figures.

**Induction of liver disease in mice**

To assess the contribution of WE-GP to strain-specific liver pathogenicity, we infected C57BL/6 mice intravenously (i.v.) with 2 × 10^6 p.f.u. wtARM, wtWE or rARM/WEGP. At the peak of disease, on day 8, we measured serum ALT and AST activity (Fig. 2a, b). As expected (Zinkernagel et al., 1986), wtWE infection elicited serum AST and ALT levels that ranged more than 20-fold above naive background.

![Fig. 2](http://vir.sgmjournals.org)  
**Fig. 2.** Behaviour of rARM/WEGP *in vivo* is similar to wtARM but not to wtWE. Mice were infected i.v. with 2 × 10^6 p.f.u. of the indicated viruses. (a, b) On day 8, serum AST (a) and ALT activity (b) was determined. (c–e) At the time points indicated, virus titres were determined in blood (c), liver (d) and spleen (e). Symbols/bars represent means ± SD of three mice. One representative experiment of at least two is shown.
whereas, in wtARM-infected mice, very little if any transaminase elevation was noted (≤2- to 3-fold over naive background). rARM/WEGP largely followed its parent virus ARM and caused very modest if any liver disease. Interestingly though, in multiple experiments, a trend was noted for the WE-GP-expressing virus to elicit slightly higher transaminase levels, although the differences observed were not statistically significant. This differential behaviour of the three viruses was highly reproducible, although the absolute levels of serum transaminase activity varied substantially between experiments. The hepatic parameters of disease correlated with high titre viraemia in wtWE-infected mice lasting for more than 14 days. In contrast, rARM/ARMGP- or rARM/WEGP-infected mice already showed lower virus load at day 4, and these viruses were cleared by day 9. Analogous findings were made for virus load in visceral organs. On day four, viral titres in liver were indistinguishable in wtWE-, wtARM- or rARM/WEGP-infected mice whereas, in spleen, wtWE infection reached somewhat higher initial values (Fig. 2d, e; analogous findings in kidney not shown). By day 8, however, wtARM and rARM/WEGP had been cleared to below detection levels from all organs tested, whereas wtWE titres were still high and clearance occurred only by day 12 or later. Thus, viral persistence in the liver throughout day 8 correlated with transaminase elevation in wtWE-infected animals, but the introduction of WE-GP into ARM (rARM/WEGP) was insufficient to confer this virus with the capacity of wtWE to induce liver disease. To verify and extend these findings, we inoculated mice i.v. with 2 × 10^6 p.f.u. of wtARM or rARM/WEGP or with titrated doses of wtWE (2 × 10^6, 2 × 10^5, 2 × 10^4, 2 × 10^3 or 2 × 10^2 p.f.u.). Blood was collected at various time points for determination of viraemia and serum transaminase activity. Again, wtWE-infected mice developed severe liver disease, whereas serum AST and ALT activities of wtARM- and rARM/WEGP-infected animals were only marginally over naive background (Fig. 3a; analogous observations for ALT not shown). As expected, AST and ALT values of wtWE-infected mice correlated positively with the virus dose (Fig. 3a). Moreover, 2 × 10^4 p.f.u. wtWE elicited serum transaminase activity similar to those elicited by 2 × 10^6 p.f.u. (i.e. 100-fold higher dose) of wtARM or rARM/WEGP. Also, viraemia was comparable when mice were inoculated with 2 × 10^5 p.f.u. of wtWE or with 2 × 10^6 p.f.u. wtARM or rARM/WEGP (Fig. 3b), demonstrating that WE had an about 100-fold higher capacity to elicit liver disease in mice.

**Virus propagation in cell culture**

These findings suggested that GP-mediated preferential tropism for liver cells could not fully account for the differential pathogenic potential of wtARM and wtWE. Disease correlated better with the ability of the virus to cause a protracted infection, with high virus loads in the liver but also in the other visceral organs tested. This prompted us to look into cell-type-specific replication kinetics as a potential mechanistic explanation. It is known that differential pathogenicity of LCMV strains and variants thereof is not reflected in differential replication rates in cell lines such as BHK-21 that are often used for virus stock preparation. Analogous observations were made for the viruses tested here (not shown). However, in mouse peritoneal macrophages (Fig. 4a), wtWE propagated faster than either wtARM or rARM/WEGP, which did not differ from each other. Similar differences were also noted in two independent experiments with the mouse hepatocyte line TIB-75 (Fig. 4b and data not shown). These findings suggested superior propagation capacity of wtWE in liver-resident cell types, a property that could not readily be transferred with the GP gene.

**Efficient spread of WE from Kupffer cells to neighbouring hepatocytes in vivo**

Next, we set out to search for an in vivo correlate to these strain-specific differences. Hepatocytes and Kupffer cells are in vivo next to each other, and their relative importance in propagation of LCMV strains is still not defined. To look into this question, we inoculated two different LCMV WE strains (wtWE and wtARM) as well as the WE-GP-expressing strain rARM/WEGP into C3H/HeJ mice and monitored the virus titre for up to 30 days. As expected, wtARM only showed lower virus load at day 4, and these viruses were cleared by day 9. The differential replication kinetics of the three viruses in this experiment were highly reproducible, with wtWE-infected mice lasting for more than 14 days. In contrast, rARM/ARMGP- or rARM/WEGP-infected mice already showed lower virus load at day 4, and these viruses were cleared by day 9. Analogous findings were made for virus load in visceral organs. On day four, viral titres in liver were indistinguishable in wtWE-, wtARM- or rARM/WEGP-infected mice whereas, in spleen, wtWE infection reached somewhat higher initial values (Fig. 2d, e; analogous findings in kidney not shown). By day 8, however, wtARM and rARM/WEGP had been cleared to below detection levels from all organs tested, whereas wtWE titres were still high and clearance occurred only by day 12 or later. Thus, viral persistence in the liver throughout day 8 correlated with transaminase elevation in wtWE-infected animals, but the introduction of WE-GP into ARM (rARM/WEGP) was insufficient to confer this virus with the capacity of wtWE to induce liver disease. To verify and extend these findings, we inoculated mice i.v. with 2 × 10^6 p.f.u. of wtARM or rARM/WEGP or with titrated doses of wtWE (2 × 10^6, 2 × 10^5, 2 × 10^4, 2 × 10^3 or 2 × 10^2 p.f.u.). Blood was collected at various time points for determination of viraemia and serum transaminase activity. Again, wtWE-infected mice developed severe liver disease, whereas serum AST and ALT activities of wtARM- and rARM/WEGP-infected animals were only marginally over naive background (Fig. 3a; analogous observations for ALT not shown). As expected, AST and ALT values of wtWE-infected mice correlated positively with the virus dose (Fig. 3a). Moreover, 2 × 10^4 p.f.u. wtWE elicited serum transaminase activity similar to those elicited by 2 × 10^6 p.f.u. (i.e. 100-fold higher dose) of wtARM or rARM/WEGP. Also, viraemia was comparable when mice were inoculated with 2 × 10^5 p.f.u. of wtWE or with 2 × 10^6 p.f.u. wtARM or rARM/WEGP (Fig. 3b), demonstrating that WE had an about 100-fold higher capacity to elicit liver disease in mice.
the most abundant cell types in the liver and represent the primary target of LCMV in this organ (Matloubian et al., 1993). We investigated by immunohistology the distribution of wtWE, wtARM and rARM/WEGP in these cells after infection with $2 \times 10^6$ p.f.u. of either virus i.v. At day 4 after infection, small spindle-shaped cells, probably Kupffer cells, were found predominantly to contain viral antigen. Eight days after infection, wtWE was widely distributed in large polygonal cells characteristic of hepatocytes, whereas rARM/WEGP and wtARM had been cleared from the liver (Fig. 5a). This correlated with the differences found in viral infectivity (compare with Fig. 2d). To identify the infected cell types unambiguously, we performed double-immunofluorescence analyses for LCMV antigen, combined with either the hepatocyte-specific marker HepPar1 or the macrophage/Kupffer cell marker F4/80 (representative panels are shown in Fig. 5b). At day 4 after infection, all three viruses were found predominantly in macrophages (~80% of LCMV+ cells; not shown). By day 8, however, the majority of WE-infected cells consisted of hepatocytes (~60% of LCMV+ cells; Fig. 5b), whereas rARM/WEGP and wtARM had become undetectable. Thus, distribution of rARM/WEGP in the liver was largely identical to its parent virus wtARM. Hepatitis, however, ensued only when the virus, such as wtWE, succeeded in efficiently spreading from infected Kupffer cells to neighbouring hepatocytes.

**Increased numbers of apoptotic hepatocytes in WE-infected mice**

An assessment of liver inflammation at the peak of disease (day 8) showed substantial infiltrations in all mice, independently of whether they had been infected with wtWE, rARM/ARMGP or rARM/WEGP (Fig. 6a and c, upper panel). However, the density of CD8+ T-cell infiltrates in periportal as well as in intralobular areas was significantly stronger in WE-infected mice compared with the other two groups (Fig. 6b), probably as a result of the higher local viral burden (compare Figs 2d and 5a). Thus, it seemed that, in wtWE-infected mice, not only the infected Kupffer cells but also hepatocytes expressing viral antigen became the target of CTLs (Zinkernagel et al., 1986) and/or died from viral interference (Djavani et al., 2005).

In support of this, TUNEL staining (Fig. 6c, lower panel) revealed that the livers of wtWE-infected mice contained significantly larger numbers of apoptotic hepatocytes ($2.55 \pm 1.49$ mm$^{-2}$) than those of rARM/ARMGP-infected ($0.34 \pm 0.11$ mm$^{-2}$), rARM/WEGP-infected ($0.47 \pm 0.11$ mm$^{-2}$) or of uninfected mice ($0 \pm 0$ mm$^{-2}$) (mean ± SD of three mice per group from one representative experiment; $P<0.01$ in a combined analysis of three experiments). The latter three groups, however, did not differ significantly from each other. Thus, rARM/ARMGP- and rARM/WEGP- but not WE-infected mice cleared the virus from the liver before substantial numbers of hepatocytes became infected, preventing severe damage to the parenchyma.

**Indistinguishable CD8+ T-cell responses**

LCMV-induced hepatitis in mice can be attenuated substantially by depletion of CD8+ T cells (Zinkernagel et al., 1986). Hence, differences in CTL responses could also have accounted for the differences in liver disease. Yet, primary ex vivo CTL assays against the immunodominant LCMV epitopes GP33, NP396 or GP276 performed 4, 8 and 12 days after infection with $2 \times 10^6$ p.f.u. i.v. did not reveal substantial differences between wtARM-, rARM/WEGP- and wtWE-infected mice (Fig. 7a). Enumeration of GP33- or NP396-specific CD8+ T cells by MHC class I tetramer staining and by intracellular cytokine assays for IFN-γ provided a similar picture (Fig. 7b and data not shown). Therefore, the extent of liver disease could not easily be attributed to differences in the magnitude or in the overall kinetics of the CTL response.

**The GP as a contributing determinant of severe liver disease**

The reverse-genetic system for LCMV is based on ARM, whereas the necessary reverse-genetic tools are not available for the WE strain. We therefore could not create the reverse complement of rARM/WEGP, i.e. a WE-based virus expressing ARM-GP instead of WE-GP. Hence we could not test whether the GP, although apparently not sufficient for WE-induced liver disease, was necessary and may still have contributed to the phenotype of wtWE.

However, neonatal infection with ARM typically yields variants in spleen and blood of carrier mice that are adapted to visceral organs and exhibit mutations not only in the S but also in the L segment (Ahmed et al., 1988). Recently we succeeded in isolating from a persistently infected mouse such a variant of rARM/INDG (clone 6; Fig. 8a; GenBank...
accession no. DQ458913; Supplementary Table S2) that had acquired four coding and six non-coding mutations in the L polymerase gene and a single non-coding mutation in Z, whereas the non-coding sequences were unaltered. When clone 6, serving as a donor for its L segment, was reassorted with the ARM-based S segment expressing WE-GP (i.e. the same S segment as in rARM/WEGP) by the protocol outlined in Fig. 1(a), a virus was recovered (rCl.6/WEGP; Fig. 8a) that caused liver disease of similar severity to WE (not shown). An rCl.6/ARMGP counterpart was also generated (Fig. 8a), and its propagation kinetics in BHK-21 cells were indistinguishable from those of rCl.6/WEGP (Supplementary Fig. S2, \( P > 0.05 \)), as expected. We therefore went on to analyse their behaviour in vivo in comparison with rARM/WEGP and rARM/ARMGP (Fig. 8b, c). By day 8 of the infection, the titres of the Cl.6-based viruses in blood, spleen, liver and kidney were substantially higher than those of their ARM-based counterparts (Fig. 8b). This highlighted the L ORF (harbouring the only coding differences between the Cl.6 and ARM L segments; Fig. 8a and Supplementary Table S2) as the most important determinant for virus load.

Interestingly, blood, liver and kidney of rCl.6/WEGP-infected mice exhibited significantly higher virus loads than rCl.6/ARM-infected ones, and a similar tendency was also seen in spleen. In accordance with this latter finding, liver disease as reflected in serum AST and ALT activity was significantly more severe in rCl.6/WEGP-infected mice (Fig. 8c), demonstrating a previously unknown and unexpected contributory role of the LCMV GP in the pathogenesis of murine hepatitis.

Fig. 5. Virus distribution in hepatocytes and Kupffer cells. (a) Mice were infected with \( 2 \times 10^6 \) p.f.u. of the viruses indicated. On days 4 and 8, three mice per group were sacrificed and LCMV antigen in the liver was detected by immunohistochemistry. For each mouse, multiple sections were analysed. One representative experiment of two is shown. Infected hepatocytes (arrows) and macrophages (arrowheads) are indicated. Bar, 20 \( \mu \)m. (b) Liver tissue collected 8 days after infection with \( 2 \times 10^6 \) p.f.u. wtWE i.v. was co-stained for LCMV (green) and F4/80 (red, left image) or HepPar1 (red, right image). Bar, 20 \( \mu \)m.
DISCUSSION

The present study reveals a contribution of the LCMV GP to strain-specific differences in liver pathogenicity, and our histological analysis suggests that the viral burden in hepatocytes, the substrate of disease, depends on efficient virus transmission from infected Kupffer cells. The affinity of GP for a cellular receptor(s) expressed on Kupffer cells and/or hepatocytes seems therefore a likely molecular candidate mechanism for its contributory role in hepatitis. Yet, the viral burden in hepatocytes is probably interdependent with the systemic virus load via at least two mechanisms: protracted virus clearance from other organs may ‘dilute’ the effector T cells at various sites of infection, thereby reducing virus control in the liver and facilitating virus spread from Kupffer cells to hepatocytes. In addition,
virus-induced immunosuppression owing to viral infection of the lymphoid compartment (Sevilla et al., 2000, 2004) may reduce the overall number and effector capacity of antiviral T cells. Both mechanisms could be influenced by the viral GP and, together with viral receptor interactions on Kupffer cells and hepatocytes, represent explanations for GP-related differences in murine liver pathogenicity that are not mutually exclusive.

Fig. 7. Indistinguishable CTL responses in wtWE, wtARM and rARM/WEGP infection. Mice were infected i.v. with $2 \times 10^6$ p.f.u. of the viruses indicated or were left uninfected. (a) On day 4, 8 or 12, NP396-, GP33- and GP276-specific primary ex vivo CTL activity of splenocytes was measured. (b) The frequency of GP33- or NP396-specific splenic CD8$^+$ T cells was determined on day 8 using MHC class I tetramers. Symbols and bars represent the mean $\pm$ SD of three mice (uninfected control groups contained two mice). One representative experiment of two is shown.

Fig. 8. GP determines the severity of liver disease elicited by clone 6-based viruses. (a) Schematic of viral genomes. Arrows indicate the orientation of individual ORFs. See Fig. 1 for details. Asterisks mark coding mutations in the clone 6 L segment compared with ARM (see also Supplementary Table S2). (b, c) Mice were infected i.v. with $2 \times 10^6$ p.f.u. of the viruses indicated. Eight days later, virus titres were determined in blood, liver, spleen and kidney (b) and serum AST and ALT activity were measured (c). One representative experiment of two is shown (except for spleen and kidney). Bars represent means $\pm$ SD from three mice.
The analysis of virus-specific CTL responses in rCl.6/ WEGP- or rCl.6/ARMGP-infected mice indeed revealed a modest reduction of NP396- but not of GP33-specific T-cell frequencies in the rCl.6/WEGP group (Supplementary Fig. S3), whereas epitope-specific CTL activity was affected even less. This finding could be due to GP-related differences in the immunosuppressive capacity of the two viruses (Sevilla et al., 2000, 2004) or, as an alternative and not mutually exclusive explanation, may result from a shift in T-cell epitope dominance under conditions of higher virus loads (Probst et al., 2003).

The L segment as the key determinant of disease in monkeys and guinea pigs is undisputed (Djavani et al., 2005; Riviere et al., 1985), and is also highlighted in mice by the differential behaviour of viruses carrying either the ARM or the clone 6 L segment (see Fig. 8). Our analysis of the clone 6 L segment sequence revealed four coding mutations compared with ARM, and all of them were located in the L ORF but none in Z (Supplementary Table S2). This identified the viral polymerase gene L as the key determinant responsible for the phenotypic differences between viruses expressing the clone 6 and ARM L segments.

In overwhelming LCMV infections, hepatocyte apoptosis is regularly seen (Balkow et al., In overwhelming LCMV infections, hepatocyte apoptosis is the clone 6 and ARM L segments.

Mechanisms similar to those observed here may also operate in so-called ‘concomitant hepatitis’, which can be caused by a multitude of viruses other than the classical hepatitis viruses A–E (Arai et al., 1995; Geisbert & Jahrling, 2004; Ho et al., 2005; Ishak et al., 1982; Kanda et al., 1995; Monto et al., 1981; Muraoka et al., 1998). These agents from a variety of different virus families are not known primarily for liver-related symptoms, but a proportion of patients exhibits clinical and biochemical signs of hepatitis (also described as accompanying or secondary hepatitis). Similarly, arenaviruses including LCMV strain WE are not exclusively hepatotropic (Ambrosio et al., 1990; Baize et al., 2004; Sevilla et al., 2000, 2004; and present report) but, in severe cases, hepatic involvement heralds an unfavourable outcome. The clear virus dose response of WE-induced hepatitis in mice (see Fig. 3) supports this notion, and arenavirus liver disease may indeed represent a ‘concomitant hepatitis’ rather than preferential infection of liver cells. It is clear though that, for arenaviruses as well as for other viruses causing ‘concomitant hepatitis’, the emergence of virus variants with a GP-mediated selective tropism for liver-resident cell types or a specific adaptation of the viral polymerase to these cells is an alternative possibility that cannot be excluded. In the specific case of WE infection, however, high virus load in a variety of organs other than the liver (Fig. 2) argues against a strictly organ-specific effect. Note, though, all wild-type and recombinant viruses behave as quasispecies, and we cannot rule out the possibility that unrepresentative variants therein may have accounted for some of the virus characteristics reported here.

Taken together, this reverse-genetic approach to an animal model of arenavirus hepatitis highlights the key role of the LCMV polymerase in determining strain-specific differences in liver pathogenicity, but it also identified a previously unknown contribution of the GP to pathogenicity which, by itself, is insufficient to cause disease.

ACKNOWLEDGEMENTS

We wish to thank Hans Hengartner, Rolf M. Zinkernagel, Hans Lutz and Wolfgang Brück for critical discussions, comments and support. A.B. is supported by a PhD fellowship of the Boehringer Ingelheim Fonds. This work was supported by grant no. 3100A0-104067/1 of the Swiss National Science foundation to D.D.P.

REFERENCES


Murooka, H., Tokeshi, S., Abe, H., Miyahara, Y., Uchimura, Y., Noguchi, S., Sata, M. & Tanikawa, K. (1998). Two cases of adult...
varicella accompanied by hepatic dysfunction. Kansenshogaku Zasshi 72, 418–423.


