Virus Receptors and Entry

30

Structure-based function analyses of pseudorabies virus glycoprotein H

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Membrane fusions are crucial events in pro- and eukaryotic organisms, e.g. during cell division, autophagy, endo- and exocytosis. Viruses also utilize fusion for entry into and release from host cells. In herpesviruses three conserved envelope glycoproteins are essential for penetration, cell-to-cell spread and induction of syncytia: the core fusion protein gB, and a heterodimer formed by gH and gL. Recently crystal structures of three gH homologues including the pseudorabies virus (PrV) protein have been uncovered, which revealed four highly conserved domains. The N-terminal domain I is required for gL-binding, whereas domain IV which includes a flap region covering a hydrophobic patch, is relevant for fusion activity. Domain II contains a planar β-sheet (fence), and a syntaxin-like bundle of α-helices (SLB), similar to those found in eukaryotic fusion proteins. These structures of PrV gH were altered by targeted mutations leading to helix disruption, or prevention of structural changes during fusion by introduction of cysteine pairs within or between fence, SLB, and domain III. Processing and transport of mutated gH was tested by Western blot and immunofluorescence analyses. Fusion assays in cells cotransfected with expression plasmids for PrV glycoproteins, and replication studies of PrV mutants are currently performed to determine the effects of the introduced mutations on gH function.

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Virus Receptors and Entry

40

Sequence requirements for membrane fusion mediated by Herpesvirus glycoproteins gD, gB, gH and gL

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Herpesviruses enter cells either by direct fusion with the plasma membrane or, after endocytic uptake, with the vesicle membrane. For fusion, four viral glycoproteins (g)D, gB and the gH/gL complex are required. gD is the receptor binding protein triggering fusion mediated by gB and gH/gL. Although gB shows signatures of class III fusion proteins, it is unable to mediate fusion in the absence of gH/gL. However, the role of gH/gL remains obscure. In porcine Pseudorabies Virus (PrV), gB and gH are also essential for direct viral cell-to-cell spread, while mutants lacking either gD or gL show some spread in tissue culture. We used this limited spread for reversion analysis. After several passages viral rescuants able to infect cells in the absence of either gD (PrV-gD$^-$Pass) or gL (PrV-DgLPass) could be isolated. Analysis of these mutants revealed mutations either in gB and gH (PrV-gD$^-$Pass) or in gD, gH and gB (PrV-DgLPass) indicating that lack of one fusion complex component can be compensated by changes in the remaining partners.
To test the influence of these mutations on fusion we established a transient transfection-fusion assay. Rabbit kidney cells were transfected with different combinations of plasmids expressing either wild-type glycoproteins or mutant forms and screened for syncytium formation. Results identified specific amino acids in gH and gB which either enhance or reduce the fusogenic potential. In combination with the recently published crystal structure for PrV gH and in comparison with structures for HSV-gB, these data shed more light on the molecular mechanism of the herpesviral fusion process.

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Epidemiology and Public Health

69
Phylogenetic analysis of pigeontype paramyxovirus 1 in Germany

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Question: Pigeon type paramyxovirus-1 (PPMV-1), a subgroup of genotype VI avian Paramyxovirus 1 (APMV-1), is endemic in German pigeons since it emerged in the early 1980ies. The current study investigates the epidemiology of PPMV-1 in Germany on the molecular level.
Methods: APMV-1 isolates (n=100) of the past four years between 2008 to 2012 were sequenced and compared to historical PPMV-1 sequences starting from year 1983 and analyzed phylogenetically, focusing on the F2-gene, including the proteolytic cleavage (374 bp) site.
Results: All APMV-1 isolates from pigeons clustered within the genotype VIb, which comprises PPMV-1. The isolates were heterogeneous and grouped within genotype VIb, lineage d and f, that were present already in Germany since 1987 (d) and 2000 (f). Starting in 2009 and dominating isolates since 2011, a distinct group of viruses within lineage f emerged. A re-appearance of in 2012 of strains closely related to viruses last detected in 2009 was noticed.
Conclusions: Outbreaks of paramyxovirosis in pigeons were caused by heterogeneous PPMV-1 strains of different lineages which co-circulated. No geographic lineage restriction was observed hinting to a flourish exchange of viruses across Europe. Re-introductions of genetically closely related PPMV-1 strains support the notion of multiple at least partially separated reservoir populations.

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Structure and Assembly

101

Mapping of sequences in Pseudorabies Virus pUL34 required for formation and function of the nuclear egress complex

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During herpesvirus replication capsids are assembled in the nucleus while further maturation takes place in the cytosol. Translocation of capsids through the nuclear envelope occurs by budding at the inner nuclear membrane (INM), resulting in a primary enveloped particle in the nuclear cleft followed by fusion of the primary envelope with the outer nuclear membrane. This process is controlled by the viral nuclear egress complex consisting of the type II membrane protein designated as pUL34 in the alphaherpesviruses Pseudorabies Virus (PrV) and Herpes Simplex Virus and its nuclear interaction partner pUL31. Both proteins are conserved throughout the herpesviruses and necessary for efficient nuclear egress.
To map regions within PrV pUL34 required for nuclear membrane targeting and pUL31 interaction, we constructed a series of deletion/substitution mutants. We showed that 85 C-terminal amino acids (aa), including the membrane anchor, could be functionally substituted by the C-terminal transmembrane domain of cellular lamin associated polypeptide (Lap)2ß. However, deletion of the 90 C-terminal aa abrogated function despite continuing pUL31 recruitment delineating the functionally important region to five aa. This region comprises the sequence "RQR", a motif which has been suggested to mediate INM targeting (Meyer et al., 2002). Site-specific mutation to RQG indicated that this motif more likely acts as Golgi retrieval signal since nuclear targeting still occurred. Deletion/substitution mutants in the N-terminus of pUL34demonstrated that only few aa can be deleted without loss of function. Mutagenesis of three conserved cysteines was tolerated, whereas alteration of a conserved "EY" sequence led to a non functional protein.

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Structure and Assembly

105
Full genome sequences of myxoma viruses

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Myxoma virus (MYXV) is a member of the *Leporipoxvirus* genus and causes myxomatosis in the European rabbit. For the examination of evolutionary relationships between leporipox viruses, we sequenced the genomes of six MYXV strains (four field isolates, MAV vaccine strain and ZA challenge strain) and compared them to the wild type strain Lausanne and the vaccine strain SG33. The genomes of the investigated strains differed from 147.6 kb (MAV) to 161.8 kb (Lausanne) and contained 153-171 open reading frames (ORFs). A/T contents varied from 55.6% to 56.4%. Phylogenetic analyses showed a close relationship between MYXVs, but all strains were affected by more or less extensive mutations covering 42-94 ORFs, respectively, resulting in amino acid residue (aa) substitutions, insertions, or deletions. Major differences were observed in the 31 immunomodulatory proteins (IMP). These ORFs are located within and in close proximity to the terminal inverted repeats (TIR). The MAV strain revealed mutations in 21 of these ORFs. Deletions of 14.3 kb in the left and right TIR accounted for the loss of nine IMP containing ankyrin repeats or belonging to the family of serine protease inhibitors. The field strain Munich-1 exhibited mutations in four IMP-ORFs and deletions of five IMP-ORFs. Further apparent mutations were identified in four ORFs encoding the putatively immunodominant envelope proteins M022L, M071L, M083L, and M115L in the MAV vaccine strain causing aa exchanges or protein truncation.

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Structure and Assembly

108
Membrane anchorage of pUL31 is not sufficient for nuclear egress function

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Herpesviruses assemble capsids in the nucleus while further maturation takes place in the cytosol. Translocation of capsids through the nuclear envelope occurs by vesicle-mediated transport, i.e. budding of capsids at the inner nuclear membrane, acquiring a primary envelope which is lost after fusion with the outer nuclear membrane. This pathway is mediated by the nuclear egress complex consisting of two conserved viral proteins designated in the alphaherpesviruses as pUL34, a type II membrane protein which localizes in the nuclear envelope, and its nuclear complex partner pUL31. In the absence of either protein nuclear egress is greatly impaired while expression of both proteins is sufficient for vesicle formation at the inner nuclear membrane.
We could show that the N-terminal part of pUL34 comprising the pUL31 interaction domain is sufficient to mediate nuclear egress in the presence of a membrane anchor. In addition, data indicate that pUL31 is sufficient to induce membrane curvature when linked to membranes. We therefore hypothesized that the primary function of pUL34 might be the timely recruitment of pUL31 to the budding site to induce membrane bending requiring only membrane anchorage of pUL31 for vesicle formation. To test this we added different transmembrane regions of cellular inner nuclear membrane proteins, e.g. Emerin and lamina associated polypeptide (Lap)2ß to the C-terminus of pUL31 or constructed chimeric proteins consisting of full-length pUL31 fused to different parts of pUL34. However, while vesicular structures could be observed by indirect immunofluorescence indicating that membrane-anchored pUL31 alone is indeed sufficient for vesicle formation, none of the chimeric proteins supported nuclear egress.

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Structure and Assembly

111

Characterization of structural proteins of koi herpesvirus

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Since the late 1990s a virus disease leading to mass mortality of common carp and koi has spread over major parts of the world. The causative agent was designated koi herpesvirus (KHV) or *Cyprinid herpesvirus 3*, and classified as a member of the family *Alloherpesviridae* within the order *Herpesvirales*. Up to now adequate, safe and efficacious vaccines are not available, and diagnostics are still almost limited to PCR detection of viral DNA in tissues of infected fish. Therefore we started to investigate predicted immunogenic virion proteins of KHV, and prepared monospecific rabbit antisera against ten of them. However, only the type III membrane protein pORF81, the type I membrane proteins pORF25 and pORF149, and the major capsid protein pORF92 were sufficiently abundant and immunogenic to permit unambiguous detection in western blot analyses of KHV-infected cells. Vice versa, in indirect immunofluorescence tests (IIFT) sera from KHV-infected carp and koi reacted with cells transfected with eukaryotic expression plasmids for pORF25, pORF65, pORF148, and pORF149 which represent a family of related KHV membrane proteins. Moreover, several monoclonal antibodies raised against KHV virions proved to be specific for pORF149 in IIFT of transfected cells, and in immunoelectron microscopic analyses of KHV particles. Since pORF149 obviously represents an immunorelevant envelope protein of KHV, recombinant baculoviruses permitting its overexpression in transduced vertebrate cells, as well as in infected insect cells were generated. Remarkably, pORF149 was also incorporated into pseudotyped baculovirus particles. The suitability of these tools for serological diagnostics, and for vaccination of carp will be further investigated.

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Structure and Assembly

114

Generation of monospecific sera against haemagglutinin (H) and fusion (F) proteins of Peste des Petits Ruminants virus (PPRV) for analyses of expression and intracellular transport of the PPRV-F and H- proteins

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Peste des petits ruminants (PPR), classified by the World Organisation for Animal Health (OIE) as notifiable disease, is a highly contagious disease of small ruminants and caused by Peste des petits ruminants virus (PPRV), a member of the genus morbillivirus in the family Paramyxoviridae. Sheep are less susceptible than goats and cattle are only subclinically infected. Its high morbidity (up to 90 %) and mortality (50% to 80%) can have a devastating impact on agricultural communities in developing countries. Clinical signs associated with the diseases are variable from peracute, acute, subacute to chronic forms and are influenced both by the species and the individual immunity to PPRV. The clinical picture of the disease is characterized by serous ocular and nasal discharges, pneumonia and diarrhea. Outbreaks of PPR need to be confirmed by isolation of the virus itself, detection of virus antigen (IC-ELISA), viral genomes (RT-PCR) or antibodies against the virus found in blood serum by virus neutralization test or competitive ELISA. Four genetic lineages (lineages 1-4) but only one serotype have been identified. Since available monoclonal antibodies against the envelope glycoproteins F and H failed to detect the respective proteins in immunoblots and did not react with the precursor proteins, monospecific antisera were raised in rabbits against bacterial fusion proteins containing selected epitopes. These sera proved to be suitable for comparative analysis of the maturation and intracellular transport of the F and H proteins of PPRV vaccine strain Nigeria 75/1 and PPRV Kurdistan/2011. Results will be presented and discussed.

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Diagnostic Methods

125
A portable point-of-entrance system for the detection of two trans-boundary viral diseases using recombinase polymerase amplification assay

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Trans-boundary viral diseases in livestock cause huge economic losses and constitute a serious threat worldwide. Early diagnosis of the infectious agents helps to diminish their impact by adequate outbreak management. Samples collected from animals in the field or at quarantine stations are sent long distances to the laboratory for PCR analysis because portable PCR is neither available nor suitable for on-site screening. The recombinase polymerase amplifications (RPA) assay is an isothermal DNA amplification and detection technology. In contrast to PCR, RPA is performed at a single temperature (42°C) and yield a result after only 5-15 minutes. In this study, we describe the development of a real-time RPA assay for the detection of lumpy skin (LSDV) and foot and mouth (FMDV) disease viruses.
Molecular DNA and RNA standards representing a part of the GPCR gene of LSDV and the 3D gene of FMDV were prepared. The assay sensitivity was determined by probit analysis (N=8 runs). The assay specificity was evaluated against a panel of viruses considered for differential diagnosis with LSDV and FMDV. The assays were validated using 22 skin nodule samples from LSDV-infected cattle and 110 samples including vesicular material, sera and swabs from FMD-infected animals. Results were compared to real-time PCR. In addition, the FMDV RPA was used in field during the recent FMDV outbreak in Egypt. The LSDV and FMDV RPAs were rapid (max. 15 minutes) and showed an analytical sensitivity of 179 and 1436 molecules detected, respectively. No cross reactivity with other viruses causing similar clinical pictures were observed. LSDV and FMDV RPAs sensitivity was 100% and 91%, respectively. In conclusion, LSDV and FMDV RPAs were quicker and much easier to handle in the field than real-time PCR. Thus RPA could be easily implemented to perform diagnostics at quarantine stations or farms for rapid on-site viral detection.

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Vaccines 1

180
Generation of immunogenic EBV virus like particles devoid of viral DNA

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The Epstein-Barr virus (EBV) is an oncogenic virus that has recently been gaining notoriety due to its increasing association with various cancers. It is estimated that EBV infection contributes to up to 2% of all tumours worldwide. Therefore, the generation of EBV prophylactic or therapeutic vaccine is a high priority. Recently, virus-like particles (VLPs) have emerged as attractive vaccine candidates. VLPs contain all structural proteins of the wild type viruses, but they lack the viral genome. Therefore, they cannot replicate and propagate, but they can elicit a specific immune response. In order to generate EBV VLPs, we attempted to block packaging of the virus DNA that takes place during virus replication. We have previously described an EBV mutant that lacks the DNA packaging signals, the terminal repeats (TR). This virus produces large amounts of defective particles, both VLPs and light particles (LPs). However, ΔTR particles exhibit minimal virus DNA contamination. To completely prevent DNA packaging, we constructed a series of mutants that lack proteins involved in virus maturation. We characterized the phenotype of those mutants in vitro and in particular their ability to produce DNA-free defective particles. Deletion of BBRF1 resulted in the production of restricted amounts of defective particles (VLPs/LPs), devoid of viral DNA. However, an EBV mutant that lacks BFLF1 and BFRF1A produced large amounts of DNA-free defective particles and elicited specific CD4+ T cell immune response, comparable in intensity to the one obtained with wild type controls. Therefore, defective particles produced by ΔBFLF1/BFRF1A fulfill the requirements for an effective and safe preventative vaccine.

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Cell Biology of Virus Infections (Working group)

233

Affinity-purification of RABV Polymerase Complex and Identification of Cellular Binding Partners

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Rabies Virus (RABV) is a neurotropic negative strand RNA virus that replicates in the CNS and causes acute encephalitis. The large polymerase L represents the catalytic subunit of the RABV polymerase complex and is essentially involved in virus replication. It is unknown whether cellular factors bind to L and whether virus-host interactions may influence host- or cell-type dependent RABV replication. To identify cellular proteins interacting with the viral polymerase complex, a recombinant RABV expressing N-terminally tagged L protein was successfully generated, L protein complexes were isolated from virus infected cells by affinity purification and analysed by nano-LC/MS analysis. One candidate interactor that reliably co-purified with the polymerase complex was Argininosuccinate Synthetase 1 (ASS1). As argininosuccinate synthesis can be a limiting step in antiviral NO production, interaction of viral polymerase complex with ASS1 may represent a novel mechanism of viral interference with NO synthesis. Further data indicate that RABV polymerase is also associated with the tubulin cytoskeleton. Notably, autofluorescent L protein N-terminally tagged with mCherry co-localized with and re-arranged tubulin filaments. Whereas ASS1 co-purification and tubulin co-localization suggested specific interactions with these cellular proteins, the number of cellular proteins that were specifically co-purified was remarkably low.

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Virus Vectors and Gene Therapy (Working group)

253
Selection system for rapid and efficient isolation of African swine fever virus recombinants

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African swine fever virus (ASFV), the sole member of the family Asfarviridae, genus Asfivirus, causes African swine fever in domestic pigs and wild boar. ASF is a highly contagious hemorrhagic disease with mortality rates up to 100 %. No efficacious vaccine has been obtained yet. Therefore it constitutes a major threat for pig husbandry worldwide, high-lighted particularly by the recent introduction of ASFV into Caucasian countries and the Russian Federation where it has become a large-scale epidemic involving both the domestic pig and wild boar population. The vicinity of some regions with circulating ASFV to the European Union (EU) borders (<150 km) has increased concerns about the potential economic consequences of an ASF incursion into the EU pig sector. The double stranded, up to 200 kbp ASFV DNA is not infectious per se and, therefore, isolation of recombinant viruses for molecular analyses of gene functions and generation of novel vaccine candidates requires the presence of ASFV helper virus which subsequently needs to be eliminated. With the hitherto used approaches, homogeneity of recombinant ASFV stocks usually requires seven to nine consecutive rounds of plaque purification which bears the risk of selecting viruses with unwanted second-site mutations that e.g. support replication in cell culture. To reduce significantly the number of plaque purifications, we established a procedure that permits isolation of recombinant ASFV after only two rounds of selection. To this end, an ASFV-permissive thymidine kinase (TK) -negative cell line was selected and used for the generation of an ASFV mutant in which the viral TK ORF was replaced by the ORF for GFP. Corresponding to the intended ASFV genome modification, helper virus can be eliminated by choosing an appropriate combination of cells, virus and HAT- or BudR-containing medium.

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Viral Replication 2

306

Trans-complementation studies with atypical pestiviruses provide new insights in the compatibility of pestivirus non-structural proteins

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Among the atypical pestiviruses, Bungowannah virus is the most divergent member of the genus *Pestivirus*. In former studies, we used heterologous complementation to clarify the phylogenetic relationship and to demonstrate the exchangeability of the structural proteins. Here, we analysed the functional replaceability of non-structural (NS) proteins.
Using a bovine viral diarrhea virus (BVDV) backbone, several chimeric constructs, generated by the substitution of the NS proteins p7, NS2, NS3 and NS4A were investigated. While constructs with substitutions in the NS3 and/or NS4A encoding regions were not able to replicate in transfected bovine cells, substitutions of p7 and/or NS2 resulted in autonomous replication. Interestingly, infectious chimeric virus could only be observed after replacing the p7-encoding region (vCP7_p7-Bungo), which had the lowest amino acid homology (28.1%) to the respective BVDV proteins. In contrast, BVDV chimeras expressing NS proteins of the atypical HoBi virus (vCP7_NS3-HoBi, vCP7_NS3NS4A-HoBi) grew to high virus titres. However, both the complementation of Bungowannah virus- and HoBi virus-NS2 resulted in replicons only, not able to generate infectious virus progeny.
Our data demonstrate, that in contrast to the putative viroporin p7 the compatibility of Bungowannah virus-NS2, -NS3 and -NS4A with a BVDV backbone is severely reduced compared to other atypical pestiviruses. Furthermore, especially NS2 needs additional interactions with homologous proteins to allow the generation of virus progeny.

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Vaccines 2

333
Pseudotyped NDV with Paramyxovirus 8 Surface Glycoproteins and Highly Pathogenic Avian Influenza Virus Hemagglutinin

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The capability of Newcastle disease virus (NDV) as a vaccine vector virus which conveys protection against highly pathogenic avian influenza virus (HPAIV) has already been shown. However, pre-existing NDV antibodies may impair vector virus replication, resulting in a lower immune response also against the foreign protein additionally expressed, like HPAIV hemagglutinin. The development of a pseudotyped NDV which possesses functional surface glycoproteins different from NDV could overcome this problem.

Here, we describe the construction of a pseudotyped vector NDV (PNDV-FHN$_{PMV8}$H5) which carries the fusion protein (F) as well as the hemagglutinin-neuraminidase protein (HN) of avian paramyxovirus type 8 (APMV-8) instead of the corresponding NDV proteins. Additionally, the HPAIV H5 gene was inserted between the APMV-8 F- and HN- genes in a NDV backbone derived from the lentogenic NDV strain Clone 30 as already described for other NDV/AIV recombinants. After successful virus rescue, the resulting pNDVFHN$_{PMV8}$H5 was further characterized. The expression of all three foreign genes was verified by Western blot analyses and indirect immunofluorescence. Furthermore, it could be shown that PNDVFHN$_{PMV8}$H5 replicates comparably to the parental viruses, resulting in high titers *in vitro* and *in vivo* after 96 hours. Animal experiments were carried out to study the protection from a lethal HPAIV infection of SPF chicken without (MDA-) and with maternally derived NDV antibodies (MDA+) after immunization with PNDVFHN$_{PMV8}$H5. MDA- as well as MDA+ chicken were protected from the lethal infection and virus shedding was significantly reduced.

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Vaccines 2

348

Expression of immunogenic proteins of zoonotic H1N1 influenza A virus in a pseudorabies virus vector

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The porcine alphaherpesvirus pseudorabies virus (PrV) is the causative agent of Aujeszky's disease which induces abortions in sows, high mortality rates in piglets, as well as fatalities in many other mammalian species, excluding higher primates and humans. Stably attenuated PrV live virus vaccines have been developed for control of the disease, which are also suitable as vectors for the expression of immunogenic proteins of other swine pathogens. Although pigs are barely harmed by influenza A viruses, they represent important reservoir hosts, and function as mixing vessels in which novel zoonotic viruses may arise. For the development of vectored influenza vaccines for pigs, and to investigate specific immune responses *in vivo*, the major structural proteins (hemagglutinin, neuraminidase, nucleoprotein, matrix proteins) of the recent pandemic swine origin H1N1 influenza A virus were expressed under control of human or murine cytomegalovirus immediate-early promoters after insertion into the genome of the PrV vaccine strain Bartha, which has been cloned as an infectious bacterial artificial chromosome (BAC). To enhance transgene expression, synthetic introns in the 5'-nontranslated part of the transcription units, and synthetic codon-optimized influenza virus genes were introduced. *In vitro* replication properties of the obtained PrV recombinants, as well as expression levels and localization of the heterologous proteins in infected cells and in virions were analyzed, and the immune response of pigs to hemagglutinin-expressing PrV is currently investigated. For immunological *in vitro* studies, the tested influenza virus antigens were also expressed in recombinant baculoviruses.

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Zoonoses

356

High prevalence of hepatitis E virus (HEV)-specific antibodies in German domestic pigs and in persons with direct contact to pigs – a comprehensive serosurvey

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An increasing number of autochthonous HEV infections has been reported for Germany. These cases are attributed to zoonotic transmission of HEV. Pig, wild boar, but also other mammals are considered sources of infection. However, the true prevalence of HEV-specific antibodies in German domestic pig herds as well as in humans with occupational exposure to them is not known. This study included 2,273 pig sera collected from almost all German federal states in 2011 as well as 537 sera from humans living in areas of Germany with a high pig-density. Application of HEV IgG-specific assays revealed a total seropositivity of 47.0% in pigs and of 13.8% in humans. HEV serostatus of pigs differed in relation to the geographic origin and the pigs' age: while 38.6% of fattening pigs (307/796) presented HEV-specific antibodies, 51.6% of sows (762/1,477) exhibited HEV-specific antibodies. 17.9% (54/302) of humans with occupational exposure to pigs exhibited IgG antibodies against HEV compared to 8.5% (20/235) in humans without direct exposure to pigs. In particular, young individuals (<40 years) with occupational exposure to pigs exhibited a significantly higher proportion of HEV-specific antibodies compared to young non-exposed individuals. HEV seroprevalence continuously increased with age. HEV genotype 3 RNA with high sequence identity to isolates from German wild boars and pigs was detected in 1/80 analyzed human sera. The data clearly demonstrate that a high percentage of German domestic pigs had contact to HEV. Thus, pigs probably represent the most important reservoir for human HEV infection in Germany. In addition to the consumption of raw or undercooked meat, direct contact to pigs has to be considered as another risk factor for HEV infection.

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Zoonoses

357
Complex evolution and epidemiology of Dobrava-Belgrade virus, the most pathogenic hantavirus in Europe

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Dobrava-Belgrade virus (DOBV) is an important human pathogen which causes hemorrhagic fever with renal syndrome (HFRS). The virus is hosted by mice of several species of the genus *Apodemus*. Phylogenetic analyses showed that DOBV forms distinct evolutionary lineages according to the host species.
Recently, we have proposed a subdivision of the DOBV species into 4 genotypes [1]. The different genotypes - despite their high genetic similarity - induce HFRS of different severity. The most severe clinical courses were observed in South-East Europewhere human infections by Dobrava genotype (associated with *A. flavicollis* mice) occur. The case-fatality rate (CFR) of clinical cases is 10-12%. Similar CFR was observed for HFRS caused by Sochi genotype (associated with *A. ponticus* mice) in the Black Sea coast area of European Russia. The course of HFRS due to infection by Kurkino genotype (associated with *A. agrarius* mice), dominant in Central Europe and in European Russia, is mainly mild or moderate. Saaremaa genotype (found in *A. agrarius* mice on the Estonian islandSaaremaa) infections seem to be subclinical.
In Germany, Kurkino genotype was molecularly detected in *A. agrarius* and *A. flavicollis* mice as well as in HFRS patients. Human infections are mainly mild to moderate but severe clinical courses with lung impairment were found, too.
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Zoonoses

358
Discovery of rodent coronaviruses related to major human and animal pathogens

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Coronaviruses (CoVs) are classified into four genera termed *Alpha-*, *Beta-*, *Gamma-* and *Deltacoronavirus*. In the aftermath of the SARS-CoV pandemic, numerous novel bat CoVs were described, suggesting an origin of mammalian alpha- and betacoronaviruses in bats. The *Betacoronavirus* 2a clade contains major animal and human CoVs (hCoV), including hCoV-OC43, hCoV-HKU1, bovine CoV (BCoV) and Mouse hepatitis virus (MHV). No bat ancestors have ever been detected for this CoV clade. We screened 4,820 rodent sera from Mexico, Germany, the Netherlands, Gabon, South Africa and Thailand by nested RT-PCR for CoVs. CoV RNA could be detected in 56 specimens (1.2%) from all sampling sites. Partial *RNA-dependent RNA polymerase* sequences indicated these viruses corresponded to at least 8 novel CoVs that phylogenetically clustered across the entire 2a CoV clade. Some viruses were closely related to hCoV-OC43, BCoV and MHV. Taxonomic classification was confirmed by full genome sequencing of two representative rodent viruses directly from clinical material. These data suggested an origin of the entire 2a CoV clade in rodents.

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Zoonoses

366

Polybasic cleavage site is essential for replication competent neuraminidase-negative HPAIV H5N1

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Question: The role of the hemagglutinin (HA) cleavage site of a replication competent but avirulent neuraminidase-negative highly pathogenic avian influenza virus (HPAIV) H5N1 should be investigated.
Methods: Virus recovery, growth kinetics and plaque sizes were measured. Virulence was studied by experimental infection of chicken and ferrets. Reverse genetics were used to generate defined virus constructs.
Results: Characterization of the NA-negative HPAIV revealed a loss of enzymatic activity. Reduced viral titers were observed until 48h post infection (p.i.) in the cell culture supernatant, while titers detected 72 h p.i. were similar to titers of the wild-type H5N1 virus. Plaque sizes of the NA-negative HPAIV were severely reduced by about 90% and application to chicken and ferrets characterized the virus variant as avirulent despite the presence of a polybasic HA-cleavage site. A recombinant H5-virus with the non-functional NA-segment could be generated, however, presence of the polybasic HA-cleavage site was a prerequisite for virus progeny. Attempts to generate the same recombinant virus with a monobasic cleavage site failed, while deletion of only 2 basic amino acids was tolerated.
Conclusions: Characterization of the NA-negative HPAIV H5N1 indicated a prominent impact of the neuraminidase on 'cell-to-cell-spread' that may count for the growth delay. However, monobasic HA of subtype H5 could not compensate for the neuraminidase negative phenotype, while neuraminidase negative virus - exhibiting a polybasic HA - was replication competent without any supplementation. Overall, the variant reported may facilitate further studies addressing the neuraminidase and its role in virus replication and pathogenicity.

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Zoonoses

368

Airway epithelial cell lines from bat and rodent wildlife species as a tool for the study of zoonotic viruses

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Due to improvements in molecular detection, an increasing number of viruses from wildlife have been described recently, but attempts to isolate them in cell culture have been largely unsuccessful. Moreover, experimental studies on these viruses are limited by the difficulty of rearing host species.

The epithelium of the respiratory tract plays a central role during respiratory transmission, as it is the first tissue encountered by viral particles and therefore serves as the first barrier during interspecies transmission between reservoir animals and humans. Even though several cell lines from a variety of animals have been established recently, there has been no focus to selectively culture airway epithelial cells from important reservoir species such as bats or rodents.

We established primary cells and immortalized cell lines from two rodent species, the bank vole *(Myodes glareolus)* and the hispid cotton rat *(Sigmodon hispidus)* as well as from two chiropteran species, the straw-colored fruit bat *(Eidolon helvum)* and Seba's short-tailed bat *(Carollia perspicillata)*. Cells were successfully cultured under standardized conditions and immortalized for the generation of permanent cell lines. Further characterization included confirmation of epithelial origin and permissiveness to viral infection. All four animal species are reservoir hosts of important emerging and re-emerging viruses with airborne transmission such as Old and New World Hantaviruses or Paramyxoviruses. Therefore, these cells can serve as suitable models for virus-host interaction of zoonotic viruses as well as a novel tool for virus isolation.

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Zoonoses

372

Putative avian/mammalian reassortant rotavirus isolated from a pheasant (*phasanus colchicus*)

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Group A rotaviruses (RVAs) are an important cause of diarrhoeal illness in humans as well as in mammalian and avian animal species. Previous sequence analyses indicated that avian RVAs are only distantly related to mammalian RVAs thus reflecting some degree of host specificity. However, the genome sequences of only two avian rotavirus strains were available so far.
Here, the complete genomes of RVA strain 03V0002E10 from turkey and RVA strain 10V0112H5 from pheasant were analyzed using 454 FLX deep sequencing. Most of the genome segments were closely related to avian RVAs; however, the VP4 gene of the pheasant RVA represents a novel genotype with highest sequence identities to RVAs from pigs, dogs, and humans. This grouping is also confirmed by the branching of this gene in phylogenetic trees, thus suggesting a reassortment event between a turkey rotavirus and a so far unknown mammalian rotavirus.
Avian rotaviruses have been previously detected in mammals and vice versa, and experimental creation of a reassortant turkey RVA strain containing a VP4-gene of a simian RVA has been described earlier. However, the analysis of the pheasant strain provides the first evidence for such reassortment under field conditions. The findings suggest that avian and mammalian RVAs are capable of exchanging genetic material under certain circumstances; thereby broadening the potential of genetic and antigenic variability among RVAs.

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Zoonoses

373

Transcriptome analysis of Cowpox Virus-infected human keratinocytes

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The continuous occurrence of zoonotic Cowpox Virus (CPXV) infections in Europe documents the relevance of deepening the understanding of virus-host interactions as pox viruses remain a considerable health threat.
The influence of an infection with two different CPXV rat isolates on the transcriptome of the human keratinocyte cell line HaCaT was analysed at different time points post infection using Illumina Sequencing technology. The CPXV strains examined differed significantly in their genome sequence and showed differing pathogenicity in an animal model. Since CPXV usually cause severe skin lesions in humans and animals the human keratinocyte cell line HaCaT was used as a model in this study. We could show that the relative abundance of cellular mRNA levels indicates that CPXV can alter the expression of genes relevant for keratinocyte differentiation and immunomodulation in human keratinocytes.

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Host Cell Factors and Modulation 1

392

ANP32B is a nuclear target of Hendra Virus Matrix Protein

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Membrane envelopment and budding of negative Strand RNA viruses is mainly driven by the viral matrix (M) proteins. In addition, M proteins are involved in host cell manipulation and virus RNA synthesis regulation. In spite of the importance of these functions for viral replication, knowledge about the molecular mechanisms, however, is poor so far for many viruses. Notably, for Nipah Virus (NiV), a member of the henipavirus family, nuclear shuttling of M has been described to be essential for virus budding. Potential nuclear targets of NiV M, however, have not been identified so far.

To identify cellular interactors of henipavirus M proteins, N- and C-terminally tagged Hendra Virus (HeV) M proteins were expressed in transfected cell cultures, M-containing protein complexes were isolated by affinity purification and their composition was analyzed by nano-LC/MS. Under different experimental conditions, cellular ANP32B (acidic leucine-rich nuclear phosphoprotein 32 family, member B) was identified as a component of the complex, suggesting that ANP32B either directly or indirectly interacts with HeV M protein. ANP32B is a multifunctional protein involved in cell cycle progression and cell survival. As ANP32B is involved in certain Crm1-dependent nuclear export processes, we analyzed whether nuclear shuttling of HeV M protein was affected by ANP32B expression. Indeed, overexpression of ANP32B led to nuclear accumulation of HeV M protein, whereas ANP32B-independent Crm1 export pathways were not inhibited. From these data we conclude that ANP32B is a nuclear target of HeV M protein and may participate in HeV M protein nuclear shuttling and/or in host cell manipulation by affecting cell survival or specific mRNA transport processes.

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Host Cell Factors and Modulation 1

393

Hepatitis A Virus protein 3ABC suppresses protein secretion by COPII vesicles

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The Hepatitis A Virus (HAV) is a non-enveloped (+)ssRNA picornavirus which causes liver inflammation in humans. The genome is translated into one large polyprotein, which is cleaved into the 11 single proteins by the viral protease 3C. A stable intermediate of this processing is 3ABC. The function of 3A, which contains a hydrophobic domain, is unknown, but may interact with membranes. 3B serves as VPg protein, which binds to the 5´end of the genome and acts as a primer for replication.

Previous studies showed that 3ABC associates with mitochondrial membranes and interacts with MAVS to suppress interferon synthesis. In addition, we found that 3ABC strongly reduced the amount of COPII (Sec31) vesicles and suppresses transport processes from the ER to the cell membrane. This shows that the noncytolytic release of HAV does not occur by this pathway.

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Host Cell Factors and Modulation 1

400

Functional study of human coronavirus Nsp1 proteins

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Human coronaviruses induce upper and lower respiratory tract diseases. Up to now, there are six different human coronaviruses discovered. They are HCoV-EMC isolated from a patient with acute pneumonia in Saudi Arabia in June 2012, HCoV-SARS resulting in severe acute respiratory syndrome outbreak in China in 2003, HCoV-NL63, HCoV-229E, HCoV-OC43, and HCoV-HKU1. Coronavirus Nsp1 protein is the first N-terminal protein cleaved from the orf1a polyprotein. In this study, the HCoV-EMC Nsp1 is compared with other human coronavirus Nsp1s in respect of subcellular localization, interaction with immunophilins, induction of host mRNA degradation, and stimulation on NFAT signaling.

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Host Cell Factors and Modulation 1

401

Influence of pseudorabies virus pUS2 and pUL46 on ERK expression and integrity of the nuclear envelope

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Herpesviruses assemble capsids in the nucleus, while further maturation occurs in the cytosol. The efficient translocation from the nucleus to the cytosol is crucial for virus maturation. This step is mediated by envelopment at the inner nuclear membrane and deenvelopment after fusion with the outer nuclear membrane mediated by the nuclear egress complex (NEC), consisting of the conserved proteins designated in the alphaherpesviruses herpes simplex and pseudorabies virus (PrV) as pUL31 and pUL34. In the absence of either protein, virion formation is significantly impaired but not completely blocked. The residual infectivity of the deletion mutants was used for serial passaging in rabbit kidney cells. Wild type like titers were observed after several passages and replication competent viruses could be isolated. Ultrastructural analysis revealed that in these mutants NEC mediated translocation was bypassed by direct access of capsids to the cytosol through the fragmented nuclear envelope (Klupp et al., 2011). Sequencing of the genomes of the passaged mutants identified seven congruent mutations in coding regions, including tegument proteins pUL46 and pUS2 (Grimm et al., 2012). Both proteins have been shown to act on cellular signaling pathways. To test for involvement of these proteins in herpesvirus-induced nuclear envelope breakdown (NEBD), mutants were generated. While simultaneous deletion of pUS2 and pUL46 from wild-type PrV did not greatly impair viral replication and had no influence on nuclear envelope stability, deletion of pUL46 from either passaged mutant resulted in an enhanced NEBD. We are currently testing the effect of deletion of US2 and UL46 on ERK expression and whether this effect is related to NEBD.

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Antiviral Therapy and Resistance 1

435
Successful isolation and expansion of CMV-Reactive T cells from healthy donors that retain A strong Cytotoxic Effector function

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Reactivation of latent cytomegalovirus (CMV) in the transient state of immunodeficiency after hematopoietic stem cell transplantation (HSCT) is a severe viral complication endangering leukemia therapy success. Cytomegalovirus (CMV) infections post-HSCT can be effectively controlled through the adoptive transfer of donor-derived CMV-specific T cells.
We have isolated CD8+ T cells from peripheral blood mononuclear cells in CMV seropositive, HLA-A*0201 healthy donors and stimulated weekly with DC loaded with the CMV pp65 peptide NLVPMVATV and interleukin-2.
CMV-specific CD8+ T-cell responses were detected in all 4 seropositive healthy donors. Using major histocompatibility complex-peptide tetramers, there was a low frequency of CMV-specific T-cells in the donors detected before stimulation of CD8+ T-cells. Starting with $1*10^6$ CD8+ cells the T-cell lines generated after 3 stimulations with peptide loaded DC contained $1,7*10^7$ to $1,2*10^8$ (average $7,9*10^7$) CD8+ T cells, consisting of 1.2%-96% (average 32%) NLVPMVATV-specific CD8+ T cells. Furthermore, functional analysis of the expanded CMV-specific T-cells revealed that these cells were able to produce interferon (IFN)γ and proved their specific cytotoxity by lysing up to 54,7% of CMV-peptide loaded target cells while sparing the unloaded target cells. These CMV-specific T cells were isolated and enriched using magnetic tetramer isolation. Starting with 6,2% CMV-specific CD8+ T cells the eluted positive fraction contained 83% CD8+ CMV-specific T cells. The TCR clonotype analysis showed a monoclonal pattern of TCR gene rearrangements in the separated T cell fraction.
These data provide the evidence that CMV-specific T cells can be effectively manufactured from low T cell numbers. However polyepitope peptides may be necessary to activate high numbers of CMV-specific T cells in all HLA-A2(+) donors.

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Viral Pathogenesis 1

436
The hemagglutinin, nucleoprotein and neuraminidase gene segments contribute to virulence in a chimeric H4/H5 avian influenza virus background

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Avian influenza viruses (AIV) possess segmented, negative-sense RNA genomes and belong to the family *Orthomyxoviridae*. Due to their virulence AIV are classified as low pathogenic (LP), exhibiting hemagglutinins (HA) of all known subtypes (H1-H16), or highly pathogenic (HP). HPAIV are restricted to the subtypes H5 and H7 with the main virulence determinant of a polybasic cleavage site (PCS) within the HA, which enables a proteolytic activation by ubiquitous proteases and in contrast to LPAIV are therefore capable to cause severe systemic disease in poultry. To investigate if this feature is sufficient to shift an LP to an HP phenotype in a non-H5/H7 background, we cloned A/mallard/Germany/1240/1/07 (H4N6) by reverse genetics with an artificial PCS (H4N6hp). However, the introduction of a PCS caused only a slight increase of virulence in infected chickens. Therefore, to investigate additional virulence determinants beside the PCS, we generated reassortant AIV of H4N6hp with all eight single gene segments of a H5N1 HPAIV exchanged. Reassortants exhibiting the H5N1 segments HA, neuraminidase (NA) and nucleoprotein (NP) showed increased virulence in chickens in the potency of HA, NP and NA. In concordance, reassortants with all possible combinations of these three segments in H4N6hp increased the virulence further on with lethal outcome. Therefore, besides the hemagglutinin and neuraminidase the nucleoprotein of H5N1 reveals a potent virulence determinant, at least in the given H4 genetic background.

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Viral Pathogenesis 1

437

Interference of Hepatitis B Virus with insulin-dependent signaling pathways and effects on liver regeneration and virus-associated pathogenesis

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Chronic hepatitis B virus infection causes liver fibrosis/cirrhosis and may lead to hepatocellular carcinoma (HCC). Deficiencies in liver regeneration and increased apoptosis promote the development of fibrosis/cirrhosis. One important factor for the control of liver regeneration is insulin. It was observed that HBV-expressing cells and HBV tg mice possess higher amounts of insulin receptor as compared with controls. Nevertheless, due to receptor delocalization, insulin sensitivity is decreased and insulin-dependent signaling cascades are impaired. In accordance to this, insulin stimulation of HBV-expressing cells fails to affect glucose metabolism and HBV tg mice significantly show higher serum glucose levels. Additionally, HBV activates the transcription factor Nrf2. Elevated activity of Nrf2 is associated with diminished activity of NF-kB and a proapoptotic potential in HBV tg mice. After partial hepatectomy and CCl_4 injection, HBV transgenic mice show more pronounced liver damage as determined by quantification of serum ALT levels and full length PARP. Moreover, quantification of BrdU incorporation indicates a delayed onset of proliferation in HBV tg mice after partial hepatectomy. Collectively, by its inhibitory effect on insulin signaling and hepatocyte proliferation, HBV impairs key steps that are crucial for liver regeneration.

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Viral Pathogenesis 1

438

Role of different regions of Newcastle disease virus fusion protein for its pathogenicity

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Newcastle disease virus (NDV), the causative agent of a notifiable disease of poultry, exhibits different levels of pathogenicity, dependent on the virus strain. However, the molecular determinants of NDV virulence are not fully understood.
The efficiency of proteolytic cleavage of the fusion protein (F) which is determined by presence or absence of a polybasic cleavage site, has long been considered a major determinant of NDV virulence. However, especially pigeon type paramyxovirus-1 (PPMV-1) isolates can exhibit low pathogenicity despite presence of a polybasic F cleavage site. Substitution of the genes encoding surface glycoproteins F and hemagglutinin-neuraminidase (HN) of a lentogenic (low virulence) NDV Clone 30 by those of a mesogenic (intermediate virulence) PPMV-1 (isolate R75/98) resulted in a recombinant NDV which possesses a polybasic F cleavage site (^{112}RRKKR*F^{117}), but low pathogenicity, demonstrated by an intracerebral pathogenicity index (ICPI) of 0.1. Substitution of only the Clone 30 F gene by that of PPMV-1 resulted also in a lentogenic recombinant NDV with an ICPI of 0.6, whereas the substitution of only the NDV Clone 30 sequence motif at the F cleavage site ^{112}GRQGR*L^{117} by that of PPMV-1 R75/98 ^{112}RRKKR*F^{117} resulted in a recombinant NDV with an ICPI of 1.36, indicating a mesogenic virus. The stepwise substitution of selected sequence regions of the F gene of NDV Clone 30 by those of PPMV-1 R75/98 and the characterization of the respective recombinant viruses demonstrated that the cytoplasmic tail of the F protein plays an important role in NDV pathogenicity in this context.

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Viral Pathogenesis 1

447

Implication of cyclophilin D in rubella virus-induced cell death

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Mitochondria play a critical role in mediating induction of apoptosis and necrosis in the cellular life cycle. They are also involved in several antiviral cellular response pathways. Rubella virus (RV) associates with mitochondria and alters mitochondrial functions and activity, which is mainly mediated by the viral capsid protein. In RV-infected cells cyclophilin D appears to be involved in cell death regulation, mainly in necrotic cell death induction. Cyclophilin D is a key regulator of the mitochondrial transition pore, which is involved in the mitochondria-associated cell death pathway. The cyclosporine A derivative NIM811 as a specific inhibitor of cyclophilin D had a profound effect on the cytopathogenicity of RV. The number of cells detached from the monolayer and the release of lactate dehydrogenase (LDH) were decreased by about 30%. Cyclophilin D translocates to the nucleus and accumulates in mitochondria at late stages of RV infection. Mere expression of the capsid protein was not sufficient for an altered localization of cyclophilin D. Additionally, RV infection resulted in opening of the mitochondrial transition pore. The results shown provide a deeper understanding of the virus-host interaction and the cytopathology of RV. Furthermore, research on cyclophilin D-mediated induction of apoptosis and necrosis may have significant implications for many non-infectious diseases, including Parkinson's disease, diabetes mellitus, and myocardial infarction.

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Viral Pathogenesis 1

448

Investigation of molecular determinants of host tropism of the avian-like swine influenza A (H1N1) lineage

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In 1979, an unknown avian influenza H1N1 virus crossed the species barrier to swine without reassortment and spread throughoutEuropeleading to a novel stable swine virus lineage. Remarkably in phylogenetic trees, the hemagglutinin and nucleoprotein sequences of those avian-like swine H1N1 viruses cluster with bird strains and not with the classical swine virus lineage. From that epizootic, the first available isolate is the strain A/Swine/Belgium/1/79 (H1N1) (SwBelg79). The avian virus most closely related is the isolate A/Duck/Bavaria/1/77 (H1N1) (DkBav77) which had been shown to be able to infect pigs. In this study, we aim to reveal the host determinants of avian-like swine H1N1 viruses after the species transmission from avian to swine host by construction of several reassortants in order to test their virulence and replication efficiency in swine respiratory tissue explants and pigs. To obtain those reassortants, we cloned all eight gene segments of both parent viruses and demonstrated the viability of plasmid clones in virus rescue experiments. Overall, this lineage of avian-like swine viruses can be utilized as a model of early evolution in a relevant mammalian host for influenza A viruses.

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Emerging Infections

540

Avian Bornaviruses (ABV) are widely distributed in canary birds (*Serinus canaria f. domestica*)

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Avian Bornaviruses (ABV) were identified in 2008 as the causative agent of proventricular dilatation disease (PDD) in psittacine birds. PDD-like diseases were also reported from other avian species, including canary birds. To date, nine ABV genotypes have been identified which can infect psittacine birds, wild waterfowl and canaries. The aim of this study was to survey the presence of ABV in canary birds and to investigate their pathogenic role. Our results demonstrate a wide distribution of ABV in captive canary birds in Germany. Sequence analysis identified several distinct ABV genotypes, which differ markedly from the genotypes present in psittacines and waterfowl. Some naturally ABV-infected birds expressed PDD-like gastrointestinal and neurological symptoms, while others did not show signs of disease.
Canary birds were experimentally infected with an ABV isolate originating from a canary bird that suffered from PDD. The experimentally infected birds showed seroconversion, viral shedding and a wide tissue distribution of the virus. In addition, ABV was successfully transmitted to sentinel birds kept in the same aviary. Embryonated eggs originating from ABV-infected hens contained ABV-specific RNA, but the virus was not detectable by re-isolation or immunohistochemistry. Interestingly, no clinical signs were observed in the experimentally infected birds until the end of the experiment at five months post infection.
Our results show that ABV expresses a remarkable genetic diversity and that different genotypes may be present in a broad range of different avian species. ABV is widely distributed in canary birds and thus should be considered as a potential pathogen of this and possibly additional passerine species.

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Emerging Infections

541

The severe fever with thrombocytopenia virus Gn/Gc glycoproteins are targeted by neutralizing antibodies and can use DC-SIGN as receptor for pH-dependent entry into human and animal cell lines

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Severe fever with thrombocytopenia syndrome virus (SFTSV) is a novel bunyavirus, which recently emerged in China. Infection with SFTSV is associated with case-fatality rates of up to 30% and neither antivirals nor vaccines are available at present. Development of antiviral strategies requires the elucidation of virus-host cell interactions. Here, we analyzed host cell entry of SFTSV. Employing lenti- and rhabdoviral vectors, we found that the Gn/Gc glycoproteins (Gn/Gc) of SFTSV mediate entry into a broad range of human and animal cell lines as well as human macrophages and dendritic cells. The Gn/Gc of La Crosse virus (LACV) and Rift Valley Fever Virus (RVFV), other members of the bunyavirus family, facilitated entry into an overlapping but not identical range of cell lines, suggesting that SFTSV, LACV and RVFV might differ in their receptor requirements. Entry driven by SFTSV-Gn/Gc was dependent on low pH but did not require the activity of the pH-dependent endo-/lysosomal cysteine proteases cathepsin B and L. Instead, the activity of a cellular serine protease was required for infection driven by SFTSV-Gn/Gc and LACV-Gn/Gc. Sera from convalescent SFTS patients inhibited SFTSV-Gn/Gc -driven host cell entry in a dose dependent fashion, demonstrating that the vector system employed is suitable to detect neutralizing antibodies. Finally, the C-type lectin DC-SIGN was found to serve as receptor for SFTSV-Gn/Gc driven entry into cell lines and dendritic cells. Our results provide first insights into cell tropism, receptor usage and proteolytic activation of SFTSV and will aid in the understanding of viral spread and pathogenesis.

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Emerging Infections

542

A self-recombinant hepatitis E virus derived from a chronically infected patient efficiently replicates in cell culture

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Hepatitis E is an increasingly reported disease in Germany. Studies on the replication cycle of hepatitis E virus (HEV) and development of vaccines are hampered due to the lack of efficient and robust cell culture systems for this virus.
We describe the successful isolation of HEV derived from a chronically infected transplant patient held under immunosuppressive therapy. Inoculation of a serum sample onto the human lung carcinoma cell line A549 resulted in replication of the virus as shown by RT-qPCR. The inoculated HEV strain is closely related to a wild boar-derived genotype 3 strain from Germany; however, this wild boar strain did not replicate in A549 cells. The most obvious difference between both strains was an insertion of 186 nucleotides in the hypervariable region of the patient strain, originating from the HEV ORF1 region. By passaging of the infected cells, a cell line continuously producing HEV particles could be generated as demonstrated by RT-qPCR, electron microscopy and immunohistochemistry. Infectivity of the produced virus was demonstrated by inoculation onto fresh A549 cells and two consecutive passages. The HEV strain derived from the second passage showed several point mutations scattered around the whole genome; however, the insertion was still present.
The data indicate cell culture replication of an uncommon HEV strain. Recently, cell culture isolation of two other HEV strains carrying also insertions in their hypervariable regions, but originating from human ribosomal RNA genes, have been described by other groups. Altogether these findings may indicate that tissue culture adaptation of HEV is mostly dependent on the length and position of the insertion, but only to lesser degree on the sequence itself.

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Emerging Infections

543

The neuraminidase beside the Hemagglutinin is the essential virulence determinant of H5N1 high-pathogenic avian influenza viruses in chicken

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High-pathogenic avian influenza viruses (HPAIV) are restricted to the HA serotypes H5 or H7 and evolve from low-pathogenic precursors by acquisition of a polybasic HA cleavage site (HACS). However, previous introductions of such a polybasic HACS into several low-pathogenic avian strains with the serotypes H5N1, H3N8, H9N2, and H4N6 did not lead to high virulence in chicken and therefore revealed the presence of additional virulence determinants both in the HA and the other seven gene segments. To map those virulence determinants of HPAIV, we generated several reassortants from two H5N1 strains with widely differing virulence, the low-pathogenic A/Teal/Germany/Wv632/2005 (TG05) and the high-pathogenic A/Swan/Germany/R65/06 (R65), to investigate their pathogenicity in chicken. An R65 HA reassortant of TG05 displayed a lethality of 30% indicating that beside a polybasic HACS, the HA gene alone provides high virulence. Remarkably, additional replacement of the PB2, PB1, PA, and NP genes with those from R65 did not increase the lethality any further, indicating minor relevance of the polymerase complex for high virulence of HPAIV in the chicken host. However, a mirror-inverted reassortant composed of the TG05 polymerase and NP genes but the HA, NA, M and NS genes from R65 displayed 100% lethality. Further exchanges of the R65 gene segments revealed that the HA and NA alone enable 100% lethality (albeit at a prolonged median death time) and efficient transmission to contact chickens. Remarkably, a TG05 reassortant carrying the R65 HA, M and NS but the NA of TG05, showed reduced lethality of 30% like the R65 HA reassortant. Therefore, beside an HA with polybasic HACS, the NA is the second essential virulence determinant of HPAIV in chicken.

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Emerging Infections

546
Molecular phylogeography of tick-borne encephalitis virus in Central Europe

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We recently reported the analysis of 61 TBEV sequences from the Czech Republic and Germany. This analysis raised some questions on the mode of TBEV migration in central Europe. In order to obtain a better understanding of TBEV strain movements in central Europe the E gene sequences of additionally 41 TBE viruses collected from 1953 - 2011 from Slovakia (4 sites), Austria (2 sites) and Germany (13 sites) were determined. This included 26 new TBEV isolates from ticks collected in South-East Germany from 2010 to 2011. This extended data set allowed analysis of 102 TBEV-E genes from 38 sites. Bayesian analysis revealed an evolutionary history spanning 350 years. Two main clades in the MCC tree and additional Median Joining network analysis indicate a) possible ingression of TBEV strains via red deer across the border between the Czech republic and Germany, b) an apparently continuous spread into Germany along rivers, a) sporadic possibly anthropogenic spread or spread via bird migration of TBEV from Central Europe to South Germany.

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Emerging Infections

547
Reinfection, oral exposure and immunity to Schmallenberg virus in cattle

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Question: In late 2011, Schmallenberg virus (SBV), an orthobunyavirus was discovered near the German-Dutch Border and spread rapidly to other European countries. Immunity in convalescent animals, the possibility of oral infection and the immune status after natural infection had to be investigated.
Methods: In this study, two previously infected heifers and five SBV antibody-negative calves were subcutaneously inoculated; another two animals received SBV orally. Whole blood and serum samples were taken regularly and lymphocytes from peripheral blood (PBL) were separated. The nostrils, oral cavity and rectum were swabbed and a diverse panel of tissue samples was taken at necropsy.
Results: After inoculation, viral RNA was detected in serum and blood samples of the naive cattle for several days. The seropositive animals remained negative throughout the study. Oral instillation of SBV did not lead to infection. Viral RNA was detected in faecal, oral and nasal swabs taken from some naive animals post inoculation. Immunological tests demonstrated that SBV did not replicate in bovine PBL but influenced the lymphocyte homeostasis in blood. Viral RNA persisted in the lymphoreticular system for at least 5 weeks after infection.
Conclusions: The animal experiment showed that only subcutaneous exposure of the naive animals to SBV led to infection, while seroconverted animals could not be re-infected. Viral RNA persisted in the lymphoreticular system, but was not present in PBL.

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Emerging Infections

550

Phylogenetic analysis of avian influenza H9N2 viruses from recent outbreaks in poultry and wild birds in Germany

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Question: Recently, influenza virus H9N2 infections in turkeys in Germany were reported. The subtype is known to circulate in the European wild bird population but is also endemic in poultry in Asia. The origin, transmission pathways and zoonotic potential of these strains remained unclear.
Methods: Hemagglutinin- and neuraminidase genes of recent H9N2 isolates from Germany were sequenced. Phylogenetic analyses were carried out in maximum likelihood and Bayesian frameworks. Antigenic properties were examined by hemagglutination inhibition assays (HI).
Results: H9N2 isolates from poultry and wild birds in Germany formed a monophyletic group together with further viruses of this subtype from Europe. No specific European "poultry lineage" was identified. Instead, sequences of viruses from wild birds and poultry were interspersed within the European lineage.
The European wild bird/poultry lineage was distinct from viruses circulating predominantly in poultry in Middle and Far Eastern countries of Asia.
Antigenic analysis by HI did not reveal gross differences between viruses of the European lineage.
Conclusions: Outbreaks of H9N2 infection in turkeys in Germany were caused by viruses with close phylogenetic and antigenic relationships to viruses circulating in the European wild bird population. Since available surveillance data do not point towards endemic H9N2 infections in European poultry, and H9N2 infections have been detected continuously over years in wild birds in Europe, spill-over transmission of virus from the wild bird population is likely to have caused sporadic outbreaks in poultry.
European H9N2 viruses are clearly distinct from Asian lineages. The latter also hold viruses which are capable to infect (man) and transmit among mammals (swine). Currently no data on related zoonotic potential in European H9N2 viruses is available.

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Emerging Infections

551

No evidence for klasse-/salivirus (family *Picornaviridae*) as a cause of acute diarrhea in patients in Germany

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Diarrhea is one of the main health problems of children and accounts for a large number of deaths in developing countries. The cause of diarrhea is unknown in approximately 40% and it is suggested that viral agents account for a large subset of cases of *unidentified* etiology.
Recently, two novel members of the family *Picornaviridae* were described termed klassevirus (kobu-like virusassociated withstool andsewage) and salivirus (stoolAichi-like virus). Both viruses are discussed as a novel pathogens causing diarrhea; with detection rates ranging from 2.1% - 8.6% in children with gastroenteritis and diarrhea and an IgG seroprevalence for klassevirus of 6.8% in children in the USA.
So far, klasse-/saliviruses have been described from a variety of regions including Nigeria, Tunisia, Korea, China, India, Nepal, Australia, Spain, Thailand, Japan, Bolivia, and the USA, but no data on the occurrence or clinical relevance of these two viruses in Germany exist. To analyze the role of klasse-/salivirus in patients presenting with diarrhea in Germany, we assessed a representative cohort of 938 stool samples from both adults (n= 237/938, 25.3%) and children (701/938, 74.7%), collected over one year. All samples were initially tested by real-time RT PCR for the five most common viruses causing diarrhea (adeno-, astro-, noro-, rota- and sapovirus), with positive findings in 268/938 (28.6%) of all samples. All negative samples were tested by two different nested RT PCR assays targeting the 3D region for klasse-/salivirus with diagnostic sensitivity. No klasse-/salivirus was detected. Thus it can be assumed that klasse-/salivirus does currently not seem to play a significant role as a cause of viral diarrhea in Germany.

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Plenary Lecture 4

560

A kinome screen identifies TLKs as novel modulators of gammaherpesvirus latency

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Kaposi's sarcoma-associated herpesvirus (KSHV) is linked to three human malignancies including Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. The majority of tumor cells harbor latent virus and a small percentage undergo spontaneous lytic replication. Both latency and lytic replication are important for viral pathogenesis and spread but the cellular players involved in the switch between the two viral lifecycle phases are not clearly understood. We conducted a siRNA screen targeting the cellular kinome and identified Tousled-like kinases (TLKs) as cellular kinases that control KSHV reactivation from latency. Upon treatment of latent KSHV-infected cells with siRNAs targeting TLKs, we saw robust viral reactivation. Knockdown of TLKs in latent KSHV-infected cells induced expression of viral lytic proteins and production of infectious virus. TLKs were also found to play a role in regulating reactivation from latency of another related oncogenic gammaherpesvirus, Epstein-Barr virus (EBV). Our results establish the TLKs as cellular repressors of gammaherpesviral reactivation.

Plenary Lecture 4

561

Schmallenberg virus – a novel orthobunyavirus emerging in Europe

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In 2011, signs of an infectious disease in cattle where reported which was associated with milk drop and fever in Germany and primarily with diarrhea in the Netherlands. A pool of blood samples from diseased dairy cows from a farm near the city of Schmallenberg was analyzed by metagenomics using next generation sequencing technology. Sequence fragments with a high homology to viruses of the genus *Orthobunyavirus* were detected, and the novel virus was designated as 'Schmallenberg Virus' (SBV), the first member of the Simbu serogroup in Europe. Obviously, the introduction occurred in a region which also experienced the first bluetongue virus serotype 8 cases in Central Europe in 2006, and the virus spread within 1.5 years over a wide geographical area. Subsequently, SBV-infection of pregnant sheep and cows resulted in the birth of characteristically malformed lambs and calves. Phylogeny and cross neutralisation demonstrated the closest relationship of SBV with viruses of the Sathuperi species like the Australian Douglas virus. PCR tests with pooled midges caught in fall 2011 were able to detect SBV-RNA in biting midges such as *Culicoides obsoletus* indicating vector-transmission similar to other Simbu serogroup viruses. First animal experiments with cattle and sheep resulted in a uniform but short viremic period of about 3 to 5 days. However, viral RNA could be detected in some animals more than 28 days post inoculation in the mesenteric lymphnodes. A zoonotic potential of SBV could be excluded by serological studies performed on humans exposed to SBV.
Possible ways of introduction, epidemiological data, genetic variability as well as results of the first pathogenesis studies will be presented, and the potential of metagenomics for pathogen discovery will be discussed.