2010

Confirmed Speakers

Andrea Ammon
Ruben Donis
Ervin Fodor
Illka Julkunen
Otfried Kistner
Hans-Dieter Klenk
Pagbajabyn Nymadawa
Juergen Richt
Robert G. Webster

2nd International Influenza Meeting

FluResearchNet.

12.–14. September

Organizing committee

Stephan Ludwig | Muenster
Klaus Schughart | Braunschweig
Peter Stäheli | Freiburg
Roland Zell | Jena
Gerlinde Benninger | Muenster
Vanessa Hugo | Muenster

Registration and abstract submission:

www.zoonosen.net

No registration fees will be charged.

Venue: Westfaelische Wilhelms-Universitaet Muenster, Schlossplatz 2, Muenster Germany

Nationale Forschungsplattform für Zoonosen

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Federal Ministry of Education and Research

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WELCOME NOTES

On behalf of the German research network FluResearchNet and the National Research Platform for Zoonoses it is my great pleasure to welcome you to the 2nd International Influenza Meeting. The meeting will be held from September 12-14, 2010 in Muenster, Germany.
The FluResearchNet is funded by the German Ministry of Education and Research (BMBF) and has been established in 2007 as the first nationwide research network that integrates virtually all leading laboratories in influenza research from basic science to veterinary medicine and clinical research. The FluResearchNet also integrates work at federal institutions (FLI/RKI/PEI) and the national reference center for influenza at the RKI.

In 2009 the FluResearchNet together with the "Sofortprogramm Influenza" has held its first international conference in Berlin that was a great success and helped to foster collaborative influenza research in Germany. This encouraged us to organize these conferences on a more regular basis and to even broaden the international scope.

We hope that with the invitation of outstanding keynote speakers from abroad, and the selection of both oral and poster presentations of submitted abstracts, the 2nd International Influenza Meeting will again be an interesting and successful meeting. To meet all aspects of the influenza field we have developed a programme that aims to bring together virologists, immunologists, epidemiologists, and public health specialists. The meeting has been designed to facilitate opportunities for informal discussions and exchange of ideas between representatives of different research disciplines and nations.

To continue our engagement against influenza we need further global efforts to understand viral mechanism, host-pathogen interactions, and immunological pathways. The H1N1 influenza pandemic that began in 2009 taught us that emerging diseases will continue to challenge us and defy our expectations. These experiences emphasize the urgent need for further basic and clinical research on influenza.

We are especially proud to welcome you in the city of Muenster on the occasion of this conference. Muenster is a multi-faceted city. It is a city with a spirit of science and knowledge, the City of Westphalian Peace, the capital city of bicycles and Germany's Climate Protection Capital. It is a place where urban culture, municipal diversity and first-class rural recreation intersect and have a mutually enhancing effect.

We are very much looking forward to welcome you in Muenster at the 2nd International Influenza Meeting – enjoy your stay.

Stephan Ludwig
Chair of the scientific committee

This conference is dedicated to honor Prof. Dr. Christoph Scholtissek, a pioneer in influenza virology, who celebrated his 80th birthday in Dec. 2009.

Christoph Scholtissek – a pioneer of influenza virology

The organizers wish to dedicate this conference to Prof. Dr. Christoph Scholtissek on the occasion of his 80th birthday celebration in December 2009.

Christoph Scholtissek was born in 1929 in Dortmund, Germany, and studied chemistry at the University of Mainz, Germany. After several scientific appointments in Heidelberg/Germany, Madison/WI/USA, and Napoli/Italy, he went to the Max-Planck-Institute for Virus Research in Tübingen, where he worked for 5 years with the late Werner Schäfer. In 1964 he went to the university of Giessen were he became Professor of Biochemistry and Virology at the Institute of Virology which in the following years developed to a leading institution in influenza virology in Germany and the world.

Christoph Scholtisseks scientific achievements are too numerous to list them all her. Just to mention a few, he was involved in characterization of the segmented nature of the influenza virus genome, he created a set of temperature-sensitive mutants that helped to define the function of the different viral gene products, he was first to propose the pig as a mixing vessel for creation of novel pandemic influenza virus strains, he showed that the NP of influenza viruses is dynamically phosphorylated throughout the virus life cycle and finally he was first to propose that inhibitors of cellular kinases and methyltransferases could be potential broad spectrum antivirals that would not lead to the emergence of resistant virus variants.

Christoph Scholtissek was retired in 1995, but he still proceeded with his scientific work for many more years. As a guest professor at the department of Virology and Molecular Biology, St. Jude Childrens´Research Hospital, Memphis, TN, USA, headed by Prof. Robert Webster he still worked in the laboratory and published several manuscripts.

Christoph Scholtissek received the Fritz-Merck-Preis in 1967 and the Award of the Society of Hygiene and Microbiology (DGHM) in 1980. In 2009 he was awarded with the Friedrich-Loeffler-Medal of the Society of Virology (GfV), one of the most prestigious awards for virologists in Germany.

The milestone work of Christoph Scholtissek builds the fundament of many of the studies presented in this meeting. Thus, it is more than fair to dedicate this conference to its honor.

Happy Birthday Christoph!

GENERAL INFORMATION

Scientific Committee
Stephan Ludwig, Muenster
Klaus Schughart, Braunschweig
Peter Stäheli, Freiburg
Roland Zell, Jena

Offical Language
The official language of the meeting is English. Simultaneous translation will not be provided.

Poster Presentations
Posters are to be mounted for the whole meeting between 15.00 and 21.00 hrs on Sunday afternoon, September 12. Posters are to be removed between 15.00 and 16.00 hrs on Tuesday, September 14.

Meals
Lunches will be provided in the Foyer of the University of Münster (Schloss), as indicated in the programme. Evening meals will be provided as part of the social programme. You are invited by the organizers.

Social Programme
Opening Reception on Sunday, September 12. The Opening reception will take place at the venue and will start at 19.00 hrs.
Address: **Universität Münster,** Schlossplatz 2, D-48149 Münster

The Conference Dinner will take place at A2 am Aasee. The restaurant is located directly at the Aasee – a small lake of Münster. We propose to meet at 20.00 hrs.
Address: **A2 am Aasee,** Annette-Allee 3, D-48149 Münster

Organization
FluResearchNet and National Platform for Zoonoses
c/o Institute of Molecular Virology (IMV)
Center of Molecular Biology of Inflammation (ZMBE)
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Sunday, September 12, 2010

15.00 Registration

18.00 - 22.00 Opening

18.00 **Welcome notes by the Rector of the University of Muenster Ursula Nelles**
Opening of the 2nd International Influenza Meeting by the conference president Stephan Ludwig

18.15 **Key-note lecture by Robert G. Webster**

19.00 Reception at the meeting site (food and drinks)

Monday, September 13, 2010

08.30 - 10.00 Plenary lecture: Influenza Virus in a Global Context

What the Pandemic Taught us for Seasonal Influenza – the EU Experience
Andrea Ammon, Stockholm, Sweden

Epidemiology and Virology of Influenza A(H1N1) 2009 pandemics in Mongolia
Pagbajabyn Nymadawa, Ulaanbaatar, Mongolia

10.00 Coffee break

10.30 - 12.00 Session 1: Pathogenesis I

Functional Analysis of the Influenza Virus RNA Polymerase
Ervin Fodor, Oxford, UK

Dissecting the Expression Mechanism of PB1, PB1-F2, and PB1-N40 and Their Contributions to Influenza A Virus Pathogenicity
Brett W. Jagger, Cambridge, UK

A Polymorphism in the Hemagglutinin of a Highly Pathogenic H5N1 Influenza Virus Determines Organ Tropism in Mice
Benjamin Mänz, Freiburg, Germany

Hemagglutinin Cleavage Site and Beyond: Virulence Determinants of HPAIV in HA and other Viral Genes
Jürgen Stech, Greifswald – Insel Riems

12.00 Lunch & Poster viewing

14.00 - 15.50 Session 2: Pathogenesis II

Evolution of Virulence in H5N1 Influenza Viruses
Ruben Donis, Atlanta, USA

NS-Reassortment of A/FPV/Rostock/34 (H7N1) HPAIV with H5-type HPAIV Increases Virulence in Chicken in *vitro* and *in vivo*
Silke Rautenschlein, Hannover

High Mortality in Pigs after Experimental Aerosol Infection with Pandemic (H1N1) 2009 virus
Ralf Dürrwald, Dessau-Rosslau, Germany

Antigenic Evolution of Influenza A (H3N2) Virus is Dictated by 7 Residues in the Hemagglutinin Protein
Björn Koel, Rotterdam, The Netherlands

Alveolar Macrophages Initiate GM-CSF-dependent Alveolar Epithelial Repair Processes after Pathogen-induced Acute Lung Injury
Susanne Herold, Giessen, Germany

15.50 Coffee break

16.15-18.05 Session 3: Virus cell interaction

Mechanisms of Pathogenicity and Host Adaptation of Influenza Viruses
Hans-Dieter Klenk, Marburg, Germany

From Virus Entry to Release - the Different Functions of the Phosphatidylinositol-3 Kinase During Influenza Virus Infection
Christina Ehrhardt, Muenster, Germany

Characterization of a SH3 Binding Motif Within the Nonstructural Protein 1 of Influenza B Virus
Anne Sadewasser, Berlin, Germany

The Cellular Rab11 Pathway is Involved in Late Stages of Influenza Assembly and Budding
Emily Bruce, Cambridge, UK

Differential Use of Importin-I± Isoforms Governs Cell Tropism and Host Adaptation of Influenza Virus
Gülsah Gabriel, Hamburg, Germany

20.00 Conference diner at the A2 am Aasee

Tuesday, September 14, 2010

08.30 - 10.20 Session 4: Pathogenesis III

The Role of the Pig in Pandemic Flu
Jürgen Richt, Manhattan, KS, USA

2009 Pandemic Influenza Virus: What Special for its HA and NA?
George Gao, Beijing, China

Pathogenicity of Mouse-Adapted Pandemic Influenza A Virus 2009 in BALB/C Mice
Alesia Romanovskaya, Koltsovo, Russian Federation

NS Reassortment of an H7-type HPAIV Affect its Propagation by Altering the Regulation of Viral RNA Production and Anti-Viral Host Response
Stephan Pleschka, Giessen, Germany

TMPRSS2 and TMPRSS4 Facilitate Trypsin-independent Influenza Virus Spread in Caco-2 cells
Stephan Pöhlmann, Hannover, Germany

10.20 Coffee break

11.00-12.50 Session 5: Innate Immunity

Activation and Inhibition of Innate Immune Responses by Influenza Viruses
Illka Julkunen, Helsiniki, Finland

Inhibition of the RIG-I Dependent Signalling Pathway by the Influenza B Virus NS1 Protein
Daniel Voss, Berlin, Germany

Toll-like Receptor 7 Contributes to Shaping Anti-Influenza Immune Responses and Influences the Outcome of Secondary Bacterial Pneumonia in Mice
Sabine Stegemann, Braunschweig, Germany

NP of panH1N1 Renders Avian H5N1 Viruses Insensitive to Antiviral Action of Interferon-induced Mx Proteins
Petra Zimmermann, Freiburg, Germany

The Adaptor Molecule FHL2 Differentially Regulates Cellular Innate and Host Immune Response to Influenza A Virus Infection
Carolin Nordhoff, Muenster, Germany

12.50 Lunch & Poster viewing

14.00-15.30 Session 6: Vaccines & Antivirals

Control of Seasonal and Pandemic Influenza – What can Novel Cell Culture Technologies Contribute?
Otfried Kistner, Orth/Donau, Austria

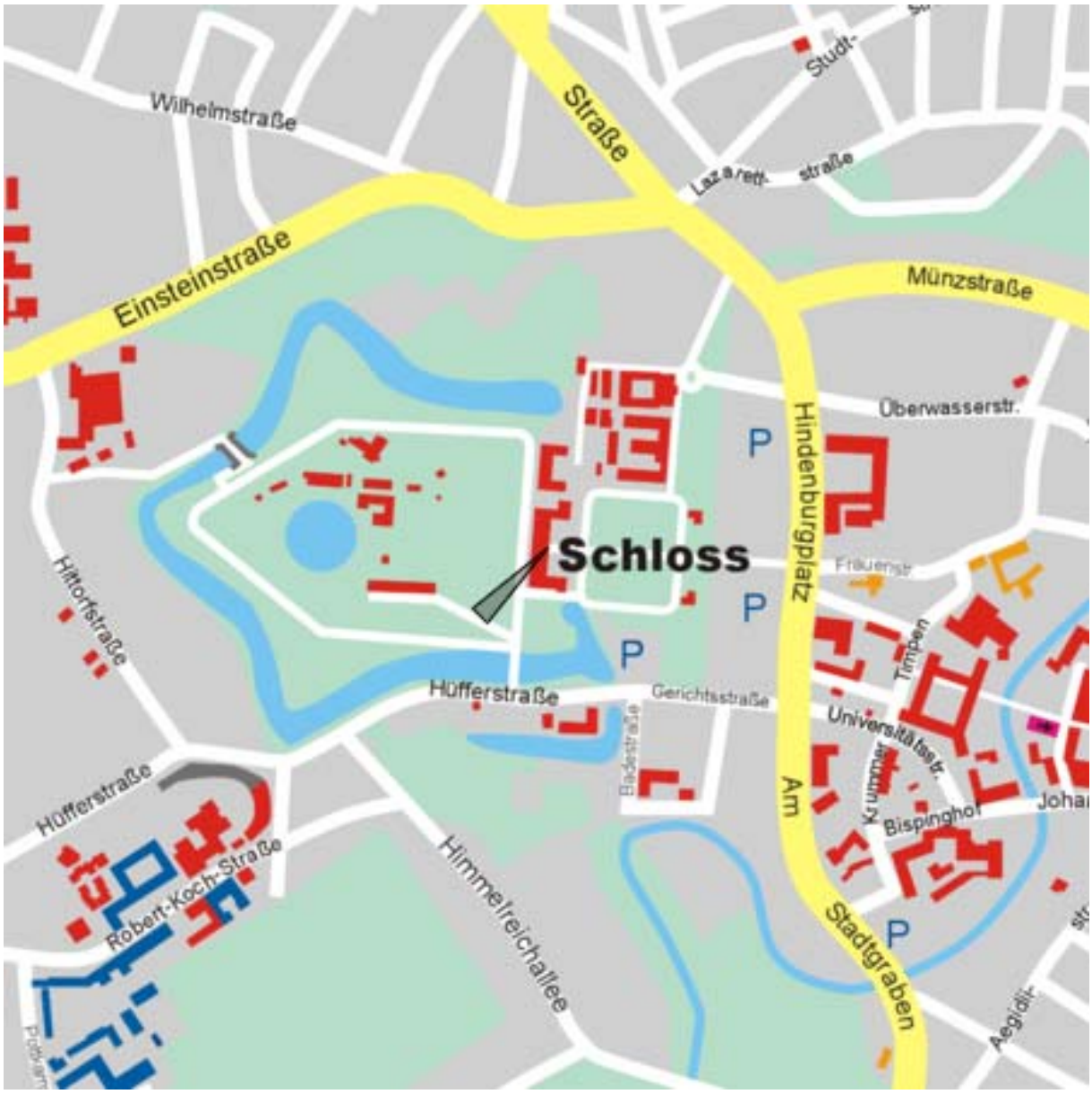
Selective Attenuation of Influenza A Viruses by Targeting the Polymerase Subunit Assembly
Martin Schwemmle, Freiburg, Germany

Identification of a 13th Influenza A Virus Protein; a Novel Splice Variant Form of the M2 Ion Channel with Important Implications for Vaccine Design
Helen Wise, Cambridge, UK

The Clinically Approved Proteasome Inhibitor PS 341 Efficiently Blocks Influenza A Virus Propagation by Establishing an Antiviral State
Sabine Eva Dudek, Muenster, Germany

15.30-16.00 Discussion and closing remarks

VENUE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Hüfferstraße
Gerichtsstraße
Universitätsstr.
Timpen
Bispinghof
Am
Stadtgraben
Robert-Koch-Straße
Himmelreichallee
Aegidii

ORAL PRESENTATIONS

Opening and plenary lecture

Sunday, September 12, 2010

KEYNOTE AT THE OPENING

The Role of Avian Influenza Viruses in the Genesis of Pandemic Influenza
Robert G. Webster, Memphis, TN, USA

Monday, September 13, 2010

Plenary lecture: Influenza Virus in a Global Context

What the Pandemic Taught us for Seasonal Influenza – the EU Experience
Andrea Ammon, Stockholm, Sweden

Epidemiology and Virology of Influenza A(H1N1) 2009 pandemics in Mongolia
Pagbajabyn Nymadawa, Ulaanbaatar, Mongolia

Session 1

Pathogenesis I

Monday, September 13, 2010

KEYNOTE SESSION 1:
Functional Analysis of the Influenza Virus RNA Polymerase
Ervin Fodor, Oxford, UK

Dissecting the Expression Mechanism of PB1, PB1-F2, and PB1-N40 and Their Contributions to Influenza A Virus Pathogenicity
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Hemagglutinin Cleavage Site and Beyond: Virulence Determinants of HPAIV in HA and other Viral Genes
Jürgen Stech et al., Greifswald – Insel Riems

Dissecting the Expression Mechanism of PB1, PB1-F2, and PB1-N40 and Their Contributions to Influenza A Virus Pathogenicity

Brett W. Jagger[1,2], Helen M. Wise[1], Jeffery Taubenberger[2] and Paul Digard[1]

[1]Department of Pathology, University of Cambridge, Cambridge, UK and [2]Laboratory of Infectious Diseases, NIAID, NIH, Bethesda, MD USA

Genome segment 2 of the influenza A virus (IAV) usually encodes three polypeptides: PB1, PB1-F2, and PB1-N40. PB1, expressed from AUG1, is the core constituent of the viral polymerase, while the variably expressed PB1-F2, expressed from AUG4, has been suggested to play pro-apoptotic and pro-inflammatory roles. The recently described PB1-N40 is expressed from the highly conserved AUG5, but its function has yet to be elucidated. Thus the segment 2-encoded proteins have essential and accepted functions in viral replication and pathogenesis, as well as emerging and unknown roles, highlighting the importance of understanding the mechanisms of their translation.

As transcription from segment 2 yields only a single, unspliced mRNA, expression of these proteins is postulated to result from leaky ribosomal scanning. Supporting this hypothesis, translation of these three polypeptides was found to be strongly interdependent, with expression levels varying with the presence or absence of intervening start codons, as well as with the strength of their surrounding Kozak initiation contexts. Using reverse genetics on pandemic (1918 and 2009), seasonal H1N1, lab-adapted H1N1 (PR8) and avian IAV-derived genes, we have generated segment 2-mutant viruses with altered expression levels of PB1-F2 and/or PB1-N40 and are characterising their replication in cell culture and animal models of pathogenicity.

Importantly, previously utilized strategies of mutating AUG4 to eliminate PB1-F2 expression resulted in a dramatic overexpression of PB1-N40 in all virus backgrounds tested. Furthermore, while mutations shown to individually cause the loss of either PB1-F2 (from an inserted stop codon at position 11) or PB1-N40 (from mutation of AUG5) on the PR8 background had no or minimal effects respectively on virus replication and pathogenicity in mice, loss of PB1-F2 and overexpression of PB1-N40 lead to lower lung titres, decreased weight loss and increased mouse survival. The simplest interpretation of these results is that overexpression of PB1-N40 is deleterious to viral virulence. Our findings have important implications for the design of viral mutants aimed at understanding the contributions of segment 2 gene products to IAV pathogenicity.

Key words: PB1, translation, pathogenesis

A POLYMORPHISM IN THE HEMAGGLUTININ OF A HIGHLY PATHOGENIC H5N1 INFLUENZA VIRUS DETERMINES ORGAN TROPISM IN MICE

Mänz B.[1,2], Matrosovich, M.[2], Bovin N.[3] and Schwemmle, M.[1]

[1]University of Freiburg, Department of Virology, 79104 Freiburg, Germany
[2]University of Marburg, Department of Virology, 35043 Marburg, Germany
[3]RAS, Shemyakin Institute of Bio-organic Chemistry, 117997 Moscow, Russia

Although there is no evidence for human-to-human transmission of H5N1 viruses, positive selection of hemagglutinin (HA) variants in infected patients reflects ongoing adaptation processes that may eventually support spread within the human population. However, little is known about the consequences of these amino acid changes in HA. By analyzing a virus isolate *A/Thailand/KAN-1/2004 (KAN-1)* from an H5N1-infected patient, we identified two major virus populations with a single polymorphism at the receptor binding site of HA (HA_{222K} and HA_{222E}). While 222K represents a characteristic avian-virus-like amino acid, 222E was suggested to be the result of a positive selection in the lung of this patient. Infection of mice with the mixture of these variants revealed replication of both virus populations in the lung, whereas only the HA_{222K} variant was found in the brain. Studies in mice infected with recombinant KAN-1 viruses encoding either HA_{222E} or HA_{222K} confirmed that each virus variant replicated efficiently in the lung, although significantly higher titers were observed for rKAN-1 HA_{222E}. At early time points after infection only rKAN-1 HA_{222K} was detectable in the brain. Surprisingly, at later time points an increase in brain titers of rKAN-1 HA_{222E}-infected animals was observed. Sequence analysis revealed that in all cases a reversion to HA_{222K} had occurred. Receptor binding properties of rKAN-1 HA_{222E} showed overall reduced binding affinity for synthetic sialylglycopolymers except one tested analogue (Su-Sle^x) to which binding was enhanced. Thus, positive selection at the HA receptor binding sites of H5N1 viruses can increase the replication efficiency in the lung and influence organ tropism.

HEMAGGLUTININ CLEAVAGE SITE AND BEYOND: VIRULENCE DETERMINANTS OF HPAIV IN HA AND OTHER VIRAL GENES

Stech J.[1], Stech O.[1], Veits J.[1], Weber, S.[1], Bogs J.[1], Gohrbandt S.[1], Hundt J.[1], Breithaupt A.[2], Teifke J. C.[2], and Mettenleiter T. C.[1]

Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institutes for [1]Molecular Biology and [2]Infectology, Südufer 10, 17493 Greifswald-Insel Riems, Germany.

Highly pathogenic avian influenza viruses (HPAIV) differ from all other strains by a polybasic cleavage site in their hemagglutinin (HA) and carry an HA with serotypes H5 or H7 only. In our investigations, we studied the ability of three low-pathogenic avian strains with the subtypes H3N8, H5N1 or H9N2 to transform into HPAIV after introduction of a polybasic HA cleavage site. HPAIV originate from LPAIV, thus, LPAI H5N1 strains have to be considered potential precursors. Furthermore, H9N2 strains are also of particular relevance as they became wide-spread across several countries and have been transmitted to humans.
In contrast to their parent viruses, all polybasic cleavage HA site mutants were able to form plaques and replicate in cell-culture in the absence of trypsin. Therefore, *in-vitro* they resemble an HPAIV. However in chicken, they did not display high virulence. The H3 cleavage site mutants led only to few temporary clinical symptoms in some chickens accompanied with cloacal shedding, whereas the H5 and H9 cleavage site mutants led to temporary non-lethal disease in all animals inoculated. However, a reassortant from LPAIV H5N1 with the HA gene from an HPAIV, displayed a lethality of 30% and, furthermore, a reassortant consisting of seven HPAIV genes and the HA gene from LPAIV H5N1 with engineered cleavage site exhibited the highest lethality of 80% resembling an authentic HPAIV. Remarkably, a reassortant expressing the modified H9 HA with polybasic cleavage site and all the other H5N1 HPAIV genes was also highly pathogenic in chicken and, with an intravenous pathogenicity index of 1.23, meets the definition of an HPAIV.
Overall, these results demonstrate that acquisition of a polybasic HA cleavage site is only one necessary step for evolution of low-pathogenic strains into HPAIV. However, the H5N1 low-pathogenic strains may already have cryptic virulence potential. Beyond the polybasic cleavage site, H5N1 HPAIV carry additional virulence determinants which are located within the HA itself and in the other viral proteins. Furthermore, if the HA would be able to acquire a polybasic cleavage site and additional virulence determinants are present, HPAIV with other subtype than H5 or H7 might emerge.

Key words: H5N1, avian influenza, virulence, chicken

Session 2

Pathogenesis II

Monday, September 13, 2010

KEYNOTE SESSION 2:
Evolution of Virulence in H5N1 Influenza Viruses
Ruben Donis, Atlanta, USA

NS-Reassortment of A/FPV/Rostock/34 (H7N1) HPAIV with H5-type HPAIV Increases Virulence in Chicken *in vitro* and *in vivo*
Silke Rautenschlein et al., Hannover; Germany

High Mortality in Pigs after Experimental Aerosol Infection with Pandemic (H1N1) 2009 Virus
Ralf Dürrwald et al., Dessau-Rosslau, Germany

Antigenic Evolution of Influenza A (H3N2) Virus is Dictated by 7 Residues in the Hemagglutinin Protein
Björn Koel et al., Rotterdam, The Netherlands

Alveolar Macrophages Initiate GM-CSF-dependent Alveolar Epithelial Repair Processes after Pathogen-induced Acute Lung Injury
Susanne Herold et al., Giessen, Germany

NS-Reassortment of A/FPV/Rostock/34 (H7N1) HPAIV with H5-type HPAIV Increases Virulence in Chicken *in vitro* and *in vivo*

Henning Petersen[1], Zhongfang Wang[2], Julia Vergara-Alert[3], Ayub Darji[3], Stephan Pleschka[2], Silke Rautenschlein[1]

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[2] Institute of Medical Virology, Justus-Liebig-University Giessen, Frankfurter Str. 107, 35392 Giessen, Germany
[3] Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

Until now H7-type highly pathogenic avian influenza viruses (HPAIV) have with one exception mainly caused mild infections in humans. Reassortment with H5-type HPAIV could change their pathogenic and pandemic potential. Previous studies allow the speculation that that the nonstructural protein 1 (NS1) may change the host response and virulence of the virus in case of reassortment. The goal of this study was to compare the virus-host interactions of the wildtype HPAIV H7N1 A/FPV/Rostock/34 (FPV wt) with its reassortant carrying the NS1 genome segment of the H5N1 A/Vietnam/1203/2004 (FPV NS VN). This comparison was conducted in different chicken host systems such as tracheal organ cultures (TOC), embryonated eggs and three-week old highly susceptible specific-pathogen-free (SPF) layer-type chickens. The replication rate of the reassortant FPV NS VN in TOC was lower compared to the wild type virus and less ciliostasis was induced during the first two passages compared to the wt strain. Interestingly, the reassortant on the other hand already induced at 8 h post infection a significantly higher apoptosis rate in TOC and more interferon than the FPV wt. These differences in virulence due to the exchange of the NS1-gene were also seen after inoculation of embryonated eggs and SPF-chickens. After intravenous infection of 14-day old embryonated chicken eggs FPV NS VN induced faster hemorrhagic lesions in the internal organs than the wt-strain. These severe lesions were already observed 16 hours post infection (pi), while wt-inoculated embryos were still free of pathological changes. After intracoanal inoculation of chickens, 70 % of FPV NS VN infected birds had died already at 4 days pi, while only 20 % of the wt-inoculated birds were dead. At day 10 pi, none of the FPV NS VN-inoculated birds had survived while still 20 % of the wt-infected birds were alive. Although the virus-replication rate of the reassortant was lower as it had been already observed in TOC *in vitro*, it induced a significantly higher apoptosis rate in the respiratory tract tissues compared to the wt-infected birds. These studies clearly show that the exchange of the NS1-protein significantly affected the host immune response without increasing the replication rate of the virus. The stronger stimulation of apoptosis and interferons may explain the increase in lesions and death rate in chicken embryos and post-hatch inoculated birds.

Key words: reassortant FPV, chicken, immune response

High mortality in pigs after experimental aerosol infection with pandemic (H1N1) 2009 virus

Ralf Dürrwald[1], Michael Schlegel[1], Andi Krumbholz[2], Michaela Schmidtke[2], Sigrid Baumgarte[3], Roland Zell[2]

[1]IDT Biologika GmbH, Dessau-Roßlau; [2]Institut für Virologie und Antivirale Therapie, Universitätsklinikum Jena, Jena; [3]Institut für Hygiene und Umwelt, Hamburg

Since its first emergence in the human population in spring 2009 infections with the pandemic H1N1 virus (p[H1N1]2009) have been reported in pigs in several regions of the world. In order to investigate cross-reactivity to European swine influenza A viruses we established hyperimmunesera, postinfection sera and postvaccination sera and investigated them by hemagglutination inhibition assay and neutralisation test. Hyperimmunesera showed cross-reaction between avian-like H1N1 (avH1N1) and p[H1N1]2009 virus but not with H1N2 and H3N2 viruses. Postinfection and postvaccination sera reflected only one-way reactivity (avH1N1 → p[H1N1]2009) or no cross-reaction at all between avH1N1 and p[H1N1]2009 virus. This demonstrates that both H1N1 viruses are distantly related but antigenetically different. Therefore we developed a monovalent inactivated vaccine for pigs based on one European strain of the p[H1N1]2009 virus. In order to investigate the efficacy of the vaccine an experimental aerosol infection trial was carried out. The study included 12 vaccinated pigs (p[H1N1]2009 vaccine), 10 pigs treated with oseltamivir (Tamiflu) and 17 unvaccinated and untreated control pigs. They were 11 weeks at the age of infection. A strain of the p[H1N1]2009 virus originally isolated from a patient in Hamburg who had returned from the USA was used for the infection. Within 24 hours after infection all control pigs and the pigs treated with oseltamivir became severely ill reflected by laboured breathing affecting the entire body, pronounced flank movement, substantial snout movement and high fever (> 41 °C). Vaccinated pigs had no symptoms. Three animals of the control group showed convulsions, ataxia, tremor, signs of hypoxia and died 2 or 3 days after infection (dpi) reflecting an unusually high mortality rate for influenza of 18%. None of the vaccinated or treated pigs died. Severe lung lesions were observed in the control pigs as early as 1 dpi (> 40% lung consolidation) which further increased on day 3 after infection (to almost 50% lung consolidation). Lung lesions were less severe in pigs treated with oseltamivir and were absent or slight in vaccinated pigs. In the 60 trials on 2280 pigs using 46 different strains of European swine influenza A viruses we have carried out during the last few years we have never observed such severe illness accompanied by such strong lung lesions and mortality rates indicating that the pandemic (H1N1) 2009 virus has a greater potential of virulence than observed in the field so far.

Key words: mortality, pigs, p[H1N1]2009 virus

Antigenic evolution of influenza A (H3N2) virus is dictated by 7 residues in the hemagglutinin protein

Björn F. Koel1*, Theo M. Bestebroer1, David F. Burke2, Jan C. de Jong1, Albert D.M.E Osterhaus1, Derek J. Smith2, Ron A.M. Fouchier1

1Department of Virology, Erasmus MC, Rotterdam, The Netherlands
2Department of Zoology, University of Cambridge, Cambridge, United Kingdom

Annual influenza A virus epidemics affect approximately 5-15% of the worlds population and are responsible for an estimated 0.25 – 0.5 million deaths annually worldwide. Vaccination is the principal way to prevent influenza disease. Antibodies against the hemagglutinin (HA) protein provide protective immunity against infection and HA is therefore the main component of influenza vaccines. However, antigenic drift - the accumulation of mutations in HA as a result of population immunity - warrants the need for frequent updates of the vaccine. Differences in the antigenic phenotype of viruses can be identified by the hemagglutination inhibition assay and this assay is widely used for detection of antigenic drift variants. Since the introduction of the H3N2 subtype in the human population in 1968 at least 11 "antigenic clusters" of viruses have emerged, each of which was subsequently replaced by antigenically distinct viruses [1]. We selected representative prototype viruses for each of the antigenic clusters, which enabled us to study the molecular determinants of antigenic evolution of the H3N2 subtype viruses over a 35-year period in detail using reverse genetics techniques.
The prototype viruses were selected to reflect the amino acid consensus sequence for the antigenic cluster they represent. Amino acid substitutions involved in cluster transitions were identified by introduction of approximately 130 specific mutations in the relevant prototype virus using site-directed mutagenesis methods, and were subsequently tested by HI assay. Results were analyzed using antigenic cartography methods. Introduction of only one to three amino acid changes was sufficient to cause a transition to the subsequent antigenic cluster, and residues 145, 155, 156, 158, 159, 189 and 193 were exclusively responsible for these phenotypic changes. All but residue 193 were involved in a cluster transition more than once. In at least three cases a single substitution was sufficient to cause a cluster transition. Analysis of the residue positions in the hemagglutinin protein showed that all antigenically important residues, except 145, are positioned adjacent to each other, and form an antigenic ridge on the membrane distal periphery of the receptor binding pocket.
Our results indicate that only seven residues dictated the antigenic evolution of H3N2 subtype viruses over a 35-year period. A single substitution in one of these residues can lead to vaccine breakdown. These residues occupy a diminutive well-defined region on the HA protein. Drift variants containing substitutions in this region should therefore be closely monitored.
[1] D.J.Smith *et al.*, Science, 305, 371 (2004)

Alveolar macrophages initiate GM-CSF-dependent alveolar epithelial repair processes after pathogen-induced acute lung injury

Unkel B, Cakarova L, Högner K, Quantius J, Seeger W, Lohmeyer J, Herold S

Influenza virus (IV) infection of the distal respiratory tract frequently results in acute lung injury (ALI) or adult respiratory distress syndrome (ARDS) usually requiring mechanical ventilation and/or extracorporal membrane oxygenation (ECMO). IV-induced ALI is characterized by direct viral cytopathic effects towards type II pneumocytes, and importantly, by an unbalanced and ongoing host response, leaving a severely damaged alveolar epithelial barrier which has to be repaired in a tightly coordinated manner to reach restitutio ad integrum. Repair mechanisms of the alveolar epithelial barrier after pneumonia-induced lung damage, however, and the particular role of alveolar macrophages herein are poorly defined.

In murine *in vitro* and *in vivo* models of LPS-, *K. pneumonia*- and IV-induced lung injury we therefore investigated whether resident alveolar macrophages contributed to alveolar epithelial repair and analyzed the molecular interaction pathways involved. We evaluated macrophage-epithelial cross-talk mediators for epithelial cell proliferation in an *in vitro* co-culture system and *in vivo* comparing wild-type, GM-CSF-deficient ($GM^{-/-}$), and SPC-GM mice ($GM^{-/-}$ mice expressing an SPC-promotor regulated GM-CSF transgene). Using qRT-PCR and ELISA we show that activated alveolar macrophages stimulated alveolar epithelial cells to express growth factors, particularly GM-CSF upon co-culture. Antibody neutralization experiments revealed epithelial GM-CSF expression to be macrophage TNF-a dependent. GM-CSF elicited proliferative signalling in alveolar epithelial cells via autocrine stimulation. Notably, macrophage TNF-ạ induced epithelial proliferation in wild-type but not in GM-CSF-deficient alveolar epithelial cells as shown by [^{3}H]-thymidine incorporation and cell counting. Moreover, intra-alveolar TNF-a neutralization impaired alveolar epithelial cell proliferation in pneumonic mice *in vivo*, as investigated by flow cytometric Ki67 staining. Additionally, GM-CSF-deficient mice displayed reduced alveolar epithelial cell proliferation, sustained alveolar barrier dysfunction and respiratory failure after pathogen-induced ALI compared to wild-type mice, associated with significantly enhanced mortality.

Collectively, these findings indicate that TNF-ạ released from activated resident alveolar macrophages induces epithelial GM-CSF expression, which in turn initiates alveolar epithelial cell proliferation and contributes to restore alveolar barrier function after bacterial or viral infection.

Key words: influenza, acute lung injury, alveolar repair, GM-CSF

Session 3

Virus cell interaction

Monday, September 13, 2010

KEYNOTE SESSION 3:
Mechanisms of Pathogenicity and Host Adaptation of Influenza Viruses
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From Virus Entry to Release - the Different Functions of the Phosphatidylinositol-3 Kinase During Influenza Virus Infection
Christina Ehrhardt et al., Muenster, Germany

Characterization of a SH3 Binding Motif Within the Nonstructural Protein 1 of Influenza B Virus
Anne Sadewasser et al., Berlin, Germany

The Cellular Rab11 Pathway is Involved in Late Stages of Influenza Assembly and Budding
Emily Bruce et al., Cambridge, UK

Differential Use of Importin-I± Isoforms Governs Cell Tropism and Host Adaptation of Influenza Virus
Gülsah Gabriel, Hamburg, Germany

From Virus Entry to Release – the Different Functions of PI3K during Influenza Virus Infection

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The Phosphatidylinositol-3-kinase (PI3K) is induced upon influenza A virus (IAV) infection by diverse mechanisms and at different time points during replication. Thereby the kinase fulfils several functions in the infected cells. While the PI3K was actually shown to induce antiviral activity via the activation of the interferon-regulatory factor 3, recent results further revealed virus supportive functions of the kinase. We could show that the very early transient activation of PI3K is observed during virus attachment and is required for efficient virus uptake. In this connection we identified receptor tyrosine kinases, such as the epidermal growth factor receptor as mediator of IAV induced PI3K activity. The later, more transient PI3K activation is dependent on the expression of the viral non-structural NS1 protein and seems to inhibit premature apoptosis. It was demonstrated by us and others that PI3K activation occurs upon direct interaction of the NS1 protein to the regulatory subunits of PI3K p85 alpha and beta. Several reports proposed that two *src homology* (SH)-binding motifs within NS1 (aa89 [YXXXM] and aa164-167 [PXXP]) may mediate binding to p85 beta. Our work confirmed this observation. However, mutant viruses of the NS1 (Y89F) only showed marginal differences to wild-type viruses with regard to their replication fitness. More detailed analysis suggested that besides expression of the NS1 there are alternative virus-induced mechanisms to activate PI3K. Here we demonstrate that this additional inducer is viral RNA (vRNA), which accumulates during infection.

CHARACTERIZATION OF A SH3 BINDING MOTIF WITHIN THE NONSTRUCTURAL PROTEIN 1 OF INFLUENZA B VIRUS

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The non-structural protein 1 (B/NS1) of influenza B virus is a multifunctional virulence factor that is required for the inhibition of IFN synthesis and for efficient viral growth. We and others had previously shown that the first 93 amino acids [aa] of the 281 aa B/NS1 protein can bind to single- and double-stranded RNA and are essential for inhibition of the protein kinase R (PKR). These functions were also described for the influenza A virus NS1 protein (A/NS1), although both proteins possess less than 25 % amino acid sequence identity.
The identification of cellular factors interacting with the B/NS1 protein is important for a more detailed understanding of the virus-host relationship.
By sequence comparison we discovered a highly conserved SH3 binding motif (aa 121-127 [YPPTPGK]) within the B/NS1 protein. By the use of a TranSignal™ SH3 domain array the enzyme phospholipase C gamma 1 (PLCγ1) and the adaptor protein CrkII were identified as cellular B/NS1 interaction partners. Crk family proteins regulate diverse signalling pathways including antiviral responses due to their ability to form transduction protein complexes.
The interaction of B/NS1 with full-length CrkII and PLCγ1 proteins was confirmed by co-immunoprecipitation assays in infected cells. Amino acid substitutions within the SH3 binding motif of B/NS1 (aa 121-127 [YGGGGGG] and [YLPTLGK], respectively) decreased binding of B/NS1 mutant proteins to full-length CrkII. Thus, we concluded that the SH3 binding motif within the B/NS1 protein is essential for binding of full-length CrkII.
The influence of the B/NS1 SH3 binding motif on virus propagation was analyzed by reverse genetics. We generated and analyzed recombinant viruses containing the above-mentioned substitutions in the SH3 binding motif of B/NS1. Unexpectedly, the substitution of the whole SH3 binding motif (aa 121-127 [YGGGGGG]) led to an increased virus propagation of more than two orders of magnitude in human lung epithelial cells compared to the recombinant B/Lee/40 wildtype virus and to accelerated expression of NS1 and NP proteins during infection.
Taken together, we described a new highly conserved interaction motif within the B/NS1 protein that allows binding of cellular signalling proteins during infection and that regulates virus propagation.

Key words: B/NS1, SH3 binding motif, CrkII, PLCγ1

The cellular Rab11 pathway is involved in late stages of influenza assembly and budding

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Influenza A virus buds through the apical plasma membrane, forming enveloped virus particles that can take the shape of pleiomorphic spheres or vastly elongated filaments. For either type of virion, the factors responsible for separation of viral and cell membranes are not known. We find that cellular Rab11 (a small GTP-binding protein involved in endocytic recycling) is required to support the formation of filamentous virions, in addition to being involved in the final budding step of spherical particles. Depletion of Rab11 resulted in up to a 85-fold decrease in titre of spherical influenza virus released from cells, while similarly treated cells showed no decrease in the release of HSV. Scanning electron microscopy of Rab11 depleted 293T cells showed high densities of virus particles apparently stalled in the process of budding. Transmission electron microscopy of thin sections confirmed that Rab11 depletion in both 293Ts and A549s resulted in significant numbers of abnormally formed virus particles that had failed to pinch off from the plasma membrane. Additionally, A549s depleted of Rab11 showed a marked defect in RNP trafficking, as observed by immunofluorescence and fluorescent *in situ* hybridisation of both the nucleoprotein and individual vRNA segments. We also detected a relocalisation of endogenous Rab11 in infected A549 cells, and a high degree of colocalisation between Rab11 and NP. Furthermore, we were able to show a specific interaction between a GFP tagged, constitutively active form of Rab11 and NP in pull-down experiments. Based on these findings, we see a clear role for the Rab11 vesicular trafficking pathway in the late stages of influenza virus assembly and budding.

Key words: assembly, RNP trafficking, morphogenesis, recycling endosome

Differential use of importin-Î± isoforms governs cell tropism and host adaptation of influenza virus

Dr. Gülsah Gabriel

Influenza A viruses are a continuous threat to humans due to their ability to cross species barriers, as illustrated by the current H1N1v pandemic and the sporadic transmission of highly pathogenic avian H5N1 viruses. Interspecies transmission requires adaptation of the viral polymerase to importin-Î±, a cellular protein that mediates transport into the nucleus where transcription and replication of the viral genome takes place1. To throw light on the role of individual importin-Î± isoforms in adaptation, here we have analyzed replication, host specificity and pathogenicity of avian and mammalian influenza viruses, including human H5N1 and H1N1v isolates, in importin-Î±-silenced cell cultures and in importin-Î±-knockout mice. We found that for efficient virus replication, the polymerase subunit PB2 of avian viruses required importin-Î±3, whereas PB2 of mammalian viruses, in general, showed importin-Î±7 specificity. H1N1v replication depended on both, importin-Î±3 and -Î±7, suggesting that adaptation of this virus is still ongoing. These observations indicate that differences in the importin-Î± specificity of viral proteins are determinants of host range underlining the crucial role of the nuclear envelope in interspecies transmission.

Session 4

Pathogenesis III

Tuesday, September 14, 2010

KEYNOTE SESSION 4:
The Role of the Pig in Pandemic Flu
Jürgen Richt, Manhattan, KS, USA

2009 Pandemic Influenza Virus: What Special for its HA and NA?
George Gao, Beijing, China

Pathogenicity of Mouse-Adapted Pandemic Influenza A Virus 2009 in BALB/C Mice
Alesia Romanovskaya et al., Koltsovo, Russian Federation

NS Reassortment of an H7-type HPAIV Affect its Propagation by Altering the Regulation of Viral RNA Production and Anti-Viral Host Response
Stephan Pleschka et al., Giessen, Germany

TMPRSS2 and TMPRSS4 Facilitate Trypsin-independent Influenza Virus Spread in Caco-2 cells
Stephan Pöhlmann et al., Hannover, Germany

2009 Pandemic influenza virus: what special for its HA and NA?

George F Gao, DPhil

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The 2009 pandemic influenza seemingly spreads extremely quickly with worrisome mortalities and resembles some characteristics of the previous three pandemics (1918 Spanish-flu, 1957 Asian-flu and 1968 Hong Kong-flu). The virus was recognized as a new swine-origin H1N1 influenza A virus (S-OIV). Functional and structural characterization of both the haemagglutinin (HA) (09H1) and the neuraminidase (NA) (09N1) might give us some clues about its pathogenesis and directs the drug application. In our group both the 09H1 and the 09N1 were prepared in a baculovirus-based system and the 09N1 enzymatic activity was verified *in vitro*. By using the *in vitro* bio-sensor binding (BIACore® machine) to both the α2,3-linked sialylglycan avian and α2,6 -linked sialylglycan human receptor analogues and crystal structure of the soluble haemagglutinin 09H1, we show that the 09H1 is very similar to the 1918 pandemic HA (18H1) in overall structure and the structural modules, including the five defined antiboby(Ab)-binding epitopes and the basic patch. It binds only the α2,6-linkage human receptor with a long half-life ($t_{1/2}$) and switches to bind both the α2,3-linkage and α2,6-linkage receptors when the D225G mutation occurs, a mutation found in the later stage of the 2009 pandemic. S-OIV containing this mutation might cause severe damage in the lung as the α2,3-linkage receptor is found there.

The 09N1 crystal structure has been solved (1.9 Å) and the structure surprisingly shows a Group 2 active cavity, different from other known N1 structures which are all categorized into Group 1. The 09N1 structures in complex with substrate sialic acid, Oseltamivir (Tamiflu®) or Zanamivir (Relanza®) have also been solved at 1.8 Å, 1.7 Å and 1.9 Å respectively, showing typical binding modes and revealing the structural basis of the effectiveness of the NA-targeted drugs against the 2009 pandemic. More importantly, the newly-defined Group-1 150-loop cavity proposed as a drug target should be re-considered as it is not as common as we thought. This is the first solved NA structure derived from swine and the first complex structure with sialic acid for Group 1 members.

Related publications from our group:

1. Sun, Y., Shi, Y, Zhang, W., Li, Q., Liu, D., Vavricka, C.J., Yan, J. & Gao, G.F., 2010, *Science China Life Sciences*, **53**, 651-660.
2. Zhang, W., Qi, J., Shi, Y., Li, Q., Gao, F., Sun, Y., Lu, X., Lu, Q., Vavricka, C.J., Liu, D., Yan J. & **Gao, G.F.**, 2010, *Protein & Cell* **1**, 459-467.
3. **Gao, G.F.** & Sun, Y., 2010, *Science China Life Sciences* **53**, 151–153.

PATHOGENICITY OF MOUSE-ADAPTED PANDEMIC INFLUENZA A VIRUS 2009 IN BALB/C MICE

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Pandemic influenza A viruses which appeared in spring 2009 are continuing to circulate in the world. A high rate of human infections with novel H1N1 influenza viruses stimulated the researchers to investigate genetic basis that determines pandemic influenza virus host range and pathogenicity in mammals.

The aim of our work was to evaluate pathogenicity of mouse-adapted virus and to elucidate the genetic basis of its increased virulence in mice.

A mouse-adapted variant of the strain A/Russia/01/2009 was derived through a series of sequential lung-to-lung 8 passages. After each passage we assessed virus titers of lung homogenates and survival of infected animals. After 6 passages mice demonstrated a trend of weight loss and some of the infected mice died within 10 days post infection (dpi), indicating the appearance of mutations that influence the virulence of the strain. The 50% mouse lethal dose (MLD50) of a virus stock prepared after 8 passages was 1,775 lg TCID50/ml.

Using a BALB/c model, we compared the virulence of generated mouse-adapted variant with that of the original strain. For a given dose of virus (1000 TCID50), all mice exhibited signs of disease and lost weight progressively. All of them died between 6 and 10 dpi. In contrast, no morbidity and mortality were observed in mice infected with the same dose of parental wild-type virus. To determine the pattern of replication of the strains A/Russia/01/2009-ma and A/Russia/01/2009 in mice lungs, we infected BALB/c mice with 1000 TCID50 of the adapted strain and with 100 000 TCID50 of the wild strain. Using MDCK cells we evaluated the virus infectivity in mice lung tissue after 1, 3, 6 and 10 dpi. The infectivity of A/Russia/01/2009-ma was 1–2 lg higher than that of the wild strain after 1, 3 and 6 dpi. Furthermore, the virus A/Russia/01/2009-ma remained in the lungs of mice until the last day of observation, while the virus A/Russia/01/2009 was undetectable on day 10.

Analysis of the deduced amino acid sequences revealed that the mouse-adapted and wild strains differed from each other by 7 amino acids distributed in the proteins of ribonucleoprotein complex (PB1: V656A, PB2: V111F) and surface glycoproteins (HA (I183T, R222K) and NA: V106I, N248D, R257K).

Thus, our findings suggest that changes in hemagglutinin may optimize receptor specificity and changes in PB1 and PB2 may influence the interaction of viral polymerase components with host cellular factors resulting in lethality of pandemic influenza viruses in mice.

Key words: influenza virus, adaptation, pathogenicity

NS reassortment of an H7-type HPAIV affects its propagation by altering the regulation of viral RNA production and anti-viral host response

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Abstract
Highly pathogenic avian influenza viruses (HPAIV) with reassorted NS segments from H5- and H7-type avian virus strains placed in the genetic background of A/FPV/Rostock/34 HAPIV (H7N1) were generated by reverse genetics. Virological characterisations demonstrated that growth kinetics of the reassortant viruses differed from the wild type FPV and depended on cells of mammalian or avian origin. Surprisingly, molecular analysis revealed that the different reassortant NS segments were not only responsible for alterations in the anti-viral host response, but furthermore affected viral genome replication and transcription as well as nuclear RNP export. RNP reconstitution experiments demonstrated that the effects on accumulation of viral RNA species depended on the specific NS-segment as well as on the genetic background of the RdRp. IFN-beta expression and the induction of apoptosis were found to be inversely correlated with the magnitude of viral growth, while the NS allele, virus subtype and levels of NS1 protein expression showed no correlation. Thus, these results demonstrate that the origin of NS segment can have a dramatic effect on the replication efficiency and host range of HPAIV. Overall our data suggest that the propagation of NS reassortant influenza viruses is affected at multiple steps of the viral lifecycle as a result of the different activities of the NS1 protein on multiple viral and host functions.

TMPRSS2 and TMPRSS4 facilitate trypsin-independent influenza virus spread in Caco-2 cells

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Proteolysis of influenza virus hemagglutinin by host cell proteases is essential for viral infectivity but the proteases responsible are not well defined. Recently, we showed that engineered expression of the type II transmembrane serine proteases (TTSPs) TMPRSS2 and TMPRSS4 allows HA cleavage. Here, we analyzed whether TMPRSS2 and TMPRSS4 are expressed in influenza virus target cells and support viral spread in the absence of exogenously added protease (trypsin). We found that transient expression of TMPRSS2 and TMPRSS4 resulted in HA cleavage and trypsin-independent viral spread. Endogenous expression of TMPRSS2 and TMPRSS4 in cell lines correlated with the ability to support influenza spread in the absence of trypsin, indicating that these proteases might activate influenza virus in naturally permissive cells. Indeed, RNAi-mediated knock-down of both TMPRSS2 and TMPRSS4 in Caco-2 cells, which released fully infectious virus without trypsin treatment, markedly reduced influenza virus spread, demonstrating that these proteases were responsible for efficient proteolytic activation of HA in this cell line. Finally, TMPRSS2 was found to be co-expressed with the major receptor determinant of human influenza viruses, 2,6-linked sialic acids, in human alveolar epithelium, indicating that viral target cells in the human respiratory tract express TMPRSS2. Collectively, our results point towards an important role of TMPRSS2 and possibly TMPRSS4 in influenza virus replication and highlight the former protease as a potential therapeutic target.

Key words: hemagglutinin, protease, TMPRSS2, TMPRSS4

Session 5

Innate immunity

Tuesday, September 14, 2010

KEYNOTE SESSION 5:
Activation and Inhibition of Innate Immune Responses by Influenza Viruses
Ilkka Julkunen, Helsiniki, Finland

Inhibition of the RIG-I Dependent Signalling Pathway by the Influenza B Virus NS1 Protein
Daniel Voss et al., Berlin, Germany

Toll-like Receptor 7 Contributes to Shaping Anti-Influenza Immune Responses and Influences the Outcome of Secondary Bacterial Pneumonia in Mice
Sabine Stegemann et al., Braunschweig, Germany

NP of panH1N1 Renders Avian H5N1 Viruses Insensitive to Antiviral Action of Interferon-induced Mx Proteins
Petra Zimmermann et al., Freiburg, Germany

The Adaptor Molecule FHL2 Differentially Regulates Cellular Innate and Host Immune Response to Influenza A Virus Infection
Carolin Nordhoff et al., Muenster, Germany

Inhibition of the RIG-I dependent signalling pathway by the influenza B virus NS1 protein

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The retinoic acid inducible gene I product (RIG-I) functions as a cellular sensor detecting viral 5´-PPP-RNA during influenza virus infection and triggering the antiviral interferon response. Recently, it has been shown that activation of RIG-I requires sequential binding of RNA and unanchored polyubiquitin chains synthesized by the E3 ubiquitin ligase TRIM25. Additionally, TRIM25 facilitates ubiquitin conjugation to activate RIG-I. Previously, we and others demonstrated that the non-structural NS1 protein of influenza A viruses (A/NS1) counteracts the RIG-I-dependent interferon (IFN) induction.
Here, we elucidated the molecular mechanism on how the highly divergent influenza B virus blocks the antiviral response. Using RNAi we showed that RIG-I is essential for influenza B virus induced IFNβ production. Reporter assays of transiently transfected cells revealed that B/NS1 inhibits the RIG-I induced IFNβ promoter activity. Although B/NS1 was found in immunoprecipitable complexes with RIG-I, we could not detect a direct interaction of the two proteins using GST-pulldown assay. Interestingly, we observed reduced ubiquitination of RIG-I in response to B/NS1 expression and an interaction of B/NS1 with the E3 ubiquitin ligase TRIM25 in a bimolecular fluorescence complementation assay.
In conclusion, our data suggest that B/NS1 inhibits the RIG-I dependent signalling pathway by binding to TRIM25 acting upstream of RIG-I and thus executing an important pathogen-host interaction in the influenza virus replication cycle.

Key words: influenza B virus, NS1 protein, RIG-I, TRIM25

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Toll-like receptor 7 contributes to shaping anti-Influenza immune responses and influences the outcome of secondary bacterial pneumonia in mice

Sabine Stegemann, Sofia Dahlberg, Birgitta Henriques-Normark, Dunja Bruder, Matthias Gunzer

The innate immune system senses Influenza A virus (IAV) through different pattern-recognition receptors including Toll-like receptor (TLR) 7. TLR7-deficient hosts have been shown to successfully mount anti-IAV responses and clear infection. However, the detailed contribution of TLR7 to local immune reactions in the acute phase of infection has not been addressed so far. Furthermore, it is still unclear what role TLR7-signalling during Influenza-infection possibly plays in its immuno-compromising effect which leads to the frequent complication of enhanced susceptibility to secondary bacterial infections.
In order to characterize the role of TLR7 in anti IAV-responses, wild-type (WT) and TLR7-deficient (TLR7ko) mice were infected intranasally with Influenza virus A/PR8/34. Local airway responses were assessed with regard to cytokine and chemokine levels, recruitment of immune-cells and histology. In addition, WT and TLR7ko mice were infected with sublethal doses of *S. pneumoniae* during acute Influenza or after resolution of the viral infection and survival as well as bacterial colonization were analyzed.
Our investigations confirmed the presence of TLR7 to be negligible for the induction of potent anti-IAV immune responses. However, a role for TLR7 in shaping these was revealed by altered airway cytokine and chemokine profiles found in TLR7ko mice. In addition, absence of TLR7 led to elevated cell numbers present in the airways of infected mice, with especially elevated numbers of B and T lymphocytes. In secondary *S. pneumoniae* infection, absence of TLR7-signalling significantly influenced the course of infection. TLR7ko mice displayed delayed disease progression despite unaltered survival. In addition, TLR7 was found to influence the long-term effect of the viral infection on bacterial susceptibility. In contrast to WT mice, survival of TLR7ko animals following bacterial super-infection increased significantly when bacterial infection occurred after resolution of the IAV infection. This suggests a detrimental role for TLR7 in viral and bacterial co-infection and future investigations will aim at further characterizing anti-IAV responses in TLR7-deficient hosts and correlating alterations in these to differences in the outcome of secondary bacterial infection. This will lead to further insights regarding the well known and still challenging phenomenon of viral and bacterial co-infections.

Key words: TLR7, innate immunity, bacterial super-infection

NP of panH1N1 renders avian H5N1 viruses insensitive to antiviral action of interferon-induced Mx proteins

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Zoonotic introduction of influenza A viruses is a constant threat for the human population. Since humans are normally immunologically naïve to these newly introduced viruses, interferon-induced defence mechanisms play a crucial role in preventing virus infection and spread. We compared human and avian influenza A virus strains including pandemic H1N1 isolate A/Hamburg/05/09 (panH1N1) and the highly pathogenic H5N1 isolate A/Thailand/1/04 (KAN-1) for their sensitivities to the antiviral action of interferon-induced murine Mx1 or human MxA GTPase. Interestingly, virus isolates of avian origin, such as KAN-1, were highly sensitive to Mx1 and MxA action, whereas isolates of human origin, including panH1N1, were relatively insensitive. Substitution of the viral components in a viral polymerase assay identified the viral nucleoprotein (NP) as the main target structure of Mx action. NP of panH1N1 rendered the KAN-1 polymerase insensitive to the antiviral action of Mx1. In contrast, NP of KAN-1 conferred Mx1 sensitivity to the polymerase of panH1N1. Next, we generated reassortant KAN-1 viruses containing segment 5 of panH1N1 for infection studies. Mx1+/+ mice survived infection with wild-type KAN-1 but succumbed to infection with the reassortant virus, indicating that the viral NP determined Mx1 sensitivity also in vivo. Our findings suggest that Mx proteins may help to prevent avian influenza A viruses from crossing the species barrier to humans. They also imply that reassortment of an avian H5N1 virus with panH1N1 could result in a loss of Mx sensitivity and increased virulence for the human host.

The Adaptor Molecule FHL2 Differentially Regulates Cellular Innate and Host Immune Response to Influenza A Virus Infection

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The innate immune response of influenza virus infected cells is predominantly mediated by interferon (IFN) type I induced proteins. Expression of IFN-ß itself is initiated by accumulating viral RNA and is transmitted by different signalling cascades that feed into activation of the three transcriptional elements located in the IFN-ß promoter, AP-1, IRF3 and NF-κB. FHL2 (four and a half LIM-domain protein 2) is an adaptor molecule that associates with numerous membrane, cytosolic and nuclear proteins, regulating thereby several signalling cascades. It shuttles between membrane and nucleus, transferring extracellular signals into the nucleus, where it can also operate as a co-factor of transcription. Here we show that FHL2 is involved in regulation of at least two transcriptional elements of the IFN-ß promoter, playing an important role in the cellular innate immune response of infected cells. Consistent with this influenza A viruses replicate in FHL2 knockout cells markedly better than in wt cells. Further, rescue of these cells by ectopic expression of FHL2 reverted the virus progeny to the level of wt cells.
To proof the physiological relevance of our in vitro observations, we infected wt and FHL2 KO mice with influenza viruses. Unexpectedly, wt mice showed a more severe onset of the disease than FHL2 KO mice. Analysis of the immune status of these animals demonstrated a more efficient immune response to virus infection in FHL2 KO mice compared to wt mice.

Session 6

Vaccines & Antivirals

Tuesday, September 13, 2010

KEYNOTE SESSION 6:
Control of Seasonal and Pandemic Influenza – What can Novel Cell Culture Technologies Contribute?
Otfried Kistner, Orth/Donau, Austria

Selective Attenuation of Influenza A Viruses by Targeting the Polymerase Subunit Assembly
Martin Schwemmle et al., Freiburg, Germany

Identification of a 13th Influenza A Virus Protein; a Novel Splice Variant Form of the M2 Ion Channel with Important Implications for Vaccine Design
Helen Wise et al., Cambridge, UK

The Clinically Approved Proteasome Inhibitor PS 341 Efficiently Blocks Influenza A Virus Propagation by Establishing an Antiviral State
Sabine Eva Dudek et al., Muenster, Germany

Selective attenuation of influenza A viruses by targeting the polymerase subunit assembly

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To develop a novel attenuation strategy applicable for all influenza A viruses, we specifically targeted the highly conserved protein-protein interaction site between the viral polymerase subunits PA and PB1. We postulated that impaired binding between PA and PB1 would negatively affect trimeric polymerase complex formation, thereby reducing viral polymerase activity and replication efficiency. As a proof of concept, we introduced single or multiple amino acid substitutions into the protein-protein binding domains of either PB1 or PA, or both, which led to a substantial reduction in binding affinity and polymerase activity. Polymerase mutants with a single amino acid substitution that retained at least 3% of wild-type activity could be generated as recombinant viruses. However, if the activity dropped below 3%, only pseudo-revertants appeared, which possessed partially restored PA-PB1 binding and polymerase activity. These pseudo-revertants nonetheless displayed drastic attenuation in immunocompetent mice as well as mice lacking a functional type I interferon-β-receptor. Vaccination of mice with these mutant viruses protected from subsequent lethal challenge with wild-type virus. Thus, targeted mutation of the highly conserved protein-protein binding domains of PA and PB1 represents a novel strategy to attenuate influenza A viruses for use as live vaccines.

Identification of a 13th Influenza A Virus Protein; A novel splice variant form of the M2 ion channel with important implications for vaccine design

Wise H.M.[1], Hutchinson E.C.[2], Kang Z.H.[1], Robb N.[2], Fodor E.[2], Firth A.E. [1], Gog J.R[3]. and Digard P[1].

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Influenza A virus segment 7 encodes 2 identified proteins; M1 from unspliced transcripts and M2 from spliced mRNA2. Alternative splicing can also produce mRNA3 and mRNA4, but protein products from these transcripts have not been identified. The 97 amino-acid M2 ion channel plays important roles in virus entry and assembly and although viruses lacking M2 are viable in tissue culture, they are highly attenuated. M2 is thus a target for antiviral drugs and antibody therapies. In particular its N-terminal ectodomain is highly conserved and is currently being intensively pursued as the target for a 'universal' M2e influenza vaccine.

Using serial passage of an M2 deficient PR8 virus lacking a functional 5'-splice donor sequence followed by reverse genetics we identified a single nucleotide compensatory mutation that was necessary and sufficient to restore wild-type growth properties in the mutant. Characterisation of the pseudorevertant virus showed no restoration of mRNA2 production but instead, upregulation of mRNA4. Furthermore, polyclonal antisera detected an M2 polypeptide of apparently normal molecular weight that did not react with the ectodomain-specific monoclonal antibody 14C2 and only weakly with anti-M2e polyclonal sera. mRNA4 has been predicted to encode an internally deleted M1 polypeptide. Through a combination of genetic and biochemical approaches, including the use of specific antisera, we show that it also encodes a variant 99 amino-acid M2 protein (here designated M42) with an alternative ectodomain whose translation is initiated by the second AUG codon in the transcript. We also find that several wild type viruses normally express low levels of mRNA4 and detectable levels of M42. Furthermore, we demonstrate that production of M2 and M42 is in balance and that single nucleotide changes in the background of otherwise WT PR8 or Udorn viruses are sufficient to upregulate mRNA4/M42 expression at the expense of mRNA2/M2 production. Importantly, this includes a change previously identified as a 14C2 antibody escape mutation.

We conclude that in many viruses, segment 7 can express at least three polypeptides and that the ion channel protein is normally comprised of two closely related polypeptides: the major M2 and minor M42 polypeptides, but that single nucleotide changes to splice donor sequences can readily swap their ratio. This suggests a ready route by which influenza A virus could potentially escape from an M2e vaccine.

Key words: M2e, splicing, vaccine.

The clinically approved proteasome inhibitor PS-341 efficiently blocks influenza A virus propagation by establishing an antiviral state

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Abstract
Recently it has been shown that the proinflammatory and antiviral NF-κB pathway promotes efficient influenza virus propagation. Thus, NF-κB blockade may be a promising approach for antiviral intervention. The classical virus-induced activation of the NF-κB pathway requires proteasomal degradation of the inhibitor of NF-κB, IκB. Therefore, we hypothesized that inhibition of the proteasomal degradation of IκB should impair influenza virus replication. As most used proteasome inhibitors act unspecific and also inhibit other proteases, we chose PS-341 as a more specific acting proteasome inhibitor. PS-341 is a clinically approved anti-cancer drug also known as Bortezomib or Velcade™. Testing the antiviral efficacy of PS-341 showed a significant reduction of viral titres of the influenza A virus (IAV) strain FPV. Surprisingly, we could not observe suppression of NF-κB-signalling *in vitro* as expected, but rather monitored the opposite activity. PS-341 treatment resulted in an induction of IκB degradation and constitutive activation of NF-κB. This coincides with enhanced expression of antiviral genes such as IL-6 and, most importantly, MxA, which is a strong IFN-induced suppressor of influenza virus replication. Comparing titres of infected A549 cells and IFN-deficient Vero cells showed a strong dependency of the antiviral efficacy of PS-341 on the IFN-system. Our data are compatible with the hypothesis that PS-341 blocks IAV replication by inducing an antiviral state mediated by the NF-κB-dependent expression of antiviral acting gene products.

Key words: PS-341, NF-κB, antiviral state

POSTER PRESENTATIONS

P1

On the origin of the 1918 pandemic influenza virus

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The pandemic of 1918 was caused by an H1N1 influenza A virus, which is a negative strand RNA virus. However, little is known about the nature of its direct ancestral strains. Here we applied a broad genetic and phylogenetic analysis of a wide range of influenza virus genes, in particular the PB1 gene, to gain information about the phylogenetic relatedness of the 1918 H1N1 virus. We compared the RNA genome of the 1918 strain to many other influenza strains of different origin by several means, including relative synonymous codon usage (RSCU), effective number of codons (ENC), and phylogenetic relationship. We found that the PB1 gene of the 1918 pandemic virus had ENC values similar to the H1N1 classical swine and human viruses, but different ENC values from avian as well as H2N2 and H3N2 human viruses. Also, according to the RSCU of the PB1 gene, the 1918 virus grouped with all human isolates and "classical" swine H1N1 viruses. The phylogenetic studies of all eight RNA gene segments of influenza A viruses may indicate that the 1918 pandemic strain originated from a H1N1 swine virus, which itself might be derived from a H1N1 avian precursor, which was separated from the bulk of other avian viruses *in toto* a long time ago. The high stability of RSCU pattern of the PB1 gene indicated that the integrity of RNA structure is more important for influenza virus evolution than previously thought.

Key words: negative strand RNA virus; 1918 pandemic virus; relative synonymous codon usage (RSCU); RSCU patterns; effective number of codons (ENC); phylogenetic relationship.

P2

Antigenic variety and biological properties of influenza B viruses circulating in Russia in 2006-2010

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The period from 2006 up to May 2010 in Russian Federation was characterized by a constant participation of B viruses in epidemiological process. In 2006-2007 they comprised 19% of all influenza viruses, in 2007-2008 – 41%, in 2008-2009 – 26%, and only in 2009-2010 – less than 1%, a situation that can be clearly explained by the prevalence of pandemic A/H1N1v strain worldwide. B viruses were isolated both in MDCK cells and in chicken embryos. Moreover, B viruses isolated in cells were easily adapted to embryos, so their receptor specificity toward mammalian cells is not absolute.

In 2006-2007 epidemical season both influenza B lineages – Victoria and Yamagata circulated in Russia, although Yamagata lineage strains were isolated rarely and only in two cities. All B-Victoria isolates were B/Malaysia/2506/04-like. The 4 Yamagata viruses were similar to B/Florida/07/04.

In 2007-2008 the situation has radically changed. Yamagata lineage predominated throughout the epidemic and all the viruses isolated in this period were similar to B/Florida/07/04. Only two Victoria-like B viruses were isolated in late May 2008 and both were similar to B/Malaysia/2506/04. The re-emergence of Victoria viruses in the end of epidemical season indicated that they could be the causative epidemic agents next season.

Indeed, in 2008-2009 only B-Victoria viruses were isolated in Russia. Also, this 2008-2009 season was characterized by an antigenic drift that occurred during previous years but wasn't evident until this season. Viruses of the current years didn't react with the reference strain B/Malaysia/2506/04. New reference strains were proposed by WHO: B/Fujian-Goulou/1272/08 that cross-reacted well with Asian isolates but didn't react with any of Russian strains and B/Brisbane/60/08 which reacted up to 1-½ of homologous titer.

The 2009 pandemic has interrupted for a while the participation of B viruses in epidemiological process but still in March – May 2010 several B-Victoria viruses were isolated in Russia. All of them were B/Brisbane/60/08-like. Strikingly, we found that they have gained a new trait. Before 2010, all Victoria-like viruses didn't cross-react with equine antisera indicating that they were inhibitor-resistant. But all 2010 isolates reacted with equine antisera to very high homologous titers (more than 1:1280). Moreover, they also react with antisera to B/Fujian-Goulou/1272/08 and B/Malaysia/2506/04 which was not observed for the viruses of the previous season.

Conclusion: in 4 seasons mentioned above, two influenza B lineages superseded one another, each dominating for 1-1,5 season. Antigenic drift was registered for B Victoria viruses pointing out that a persistent process of viral evolution still proceeds towards effective host infection.

Key words: influenza B, antigenic variability, isolation systems, thermostable inhibitors

P3

Cell cultures susceptibility and proliferative response to avian, human and swine influenza viruses

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Multiple reassortment events have resulted in emergence of a new pandemic virus - an H1N1v – with altered antigenic and biological properties. The objective of our study was to compare growth characteristics of different flu viruses (e.g. swine, avian and human) on various human and animal cell cultures and to evaluate their influence on cell culture growth.

The following viruses were used in the present study: swine – A/Swine/1976/31 (H1N1); avian – A/Chicken/Kurgan/5/05 (highly pathogenic H5N1), A/Gull/Kostanai/7/07 (low pathogenic H5N1); human – A/Brisbane/59/07 (H1N1), A/Brisbane/10/07 (H3N2), A/Saint-Petersburg/5/09 (H1N1v). We have chosen 8 human and 2 animal monolayer cell cultures to investigate the comparative rate of viral replication. Animal cell cultures included MDCK (canine kidney) as a model culture for influenza virus isolation and SP (porcine kidney, in-house culture). Human cell lines comprised A549 (lung adenocarcinoma), FLECH (human embryo lung fibroblasts), ECV-304 (endothelial line), L-41 (monocytic leukemia), RD (rhabdomyosarcoma), Girardi Heart (cells of auricle), T-98 (glioblastoma) and A172 (glioblastoma). Also we checked several suspension human cell lines: hemablastoid T-lymphocytes (Jurkat), B-lymphoblastoid lines (NC-37, Raji) and histiocytes (U-937).

All tested influenza viruses replicated well in MDCK but the SP cell culture was less sensitive to infection, especially for pandemic H1N1v strain. Human monolayer cell cultures were most sensitive to avian influenza viruses. Human seasonal H1N1 and H3N2 viruses infected all human cell cultures but displayed only weak replication. Strikingly, pandemic H1N1v and swine viruses practically didn't replicate in any cell culture other than MDCK and had the narrowest range of susceptible cell cultures for infection.

Suspension cell cultures were susceptible to all influenza viruses tested but replication of influenza viruses wasn't efficient. Still, we revealed an interesting effect when inoculating these cultures with low infection doses (MOI <0,01). In this case, the clear stimulation of cell proliferation was observed, and apoptosis induction afterwards was registered. The degradation of chromatin was typically apoptotic and was seen in all cell cultures. Even in those cultures where the viral propagation wasn't visible by cytopathic effect, the infection process was ongoing which could be confirmed by the presence of NP in infected cells (seen in fluorescent assay with monoclonal antibodies). The monolayer cell cultures ECV-304 and T-98 showed the similar stimulation of cell culture proliferation when infected with low MOI. Other monolayer cell cultures didn't respond to low viral doses.

Key words: influenza, monolayer cell cultures, suspension cell cultures, viral replication.

P4

Proteolytic Activation of Avian Influenza A-Virus Hemagglutinin of the Subtype H9N2

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Influenza A-viruses of subtype H9N2 are enzootic in large parts of Asia and Europe, co-circulate with H5N1 viruses, and they share internal genes with H5N1 viruses. In addition, they are occasionally transmitted to humans, in which they cause mild respiratory diseases. Therefore, H9N2 viruses belong to the avian influenza viruses that have a pandemic potential.
The hemagglutinin (HA) of influenza virus is the major target for immune responses. The glycoprotein mediates attachment to and penetration into host cells. It is synthesized as a precursor protein HA_0 and must be cleaved by cellular proteases into the two subunits HA_1 and HA_2 to be active. HAs of high-pathogenic avian influenza viruses (HPAIV) have a polybasic cleavage site (R-X-X-R), which is recognised by ubiquitous subtilases like furin. Low-pathogenic avian influenza viruses (LPAIV) are cleaved at a single arginine by trypsin-like proteases, which are restricted to enteric or respiratory epithelia. Thus, infection is restricted to the respective organs. In general, H9N2 viruses belong to the LPAIV type. However, some HAs of avian H9N2 isolates have a multibasic cleavage sites similar to subtypes H5 and H7. The susceptibility of these HAs to proteases and their role as determinants of pathogenicity are not known.
Here, we compare two H9N2 viruses A/quail/Shantou/782/2000 (R-S-S-R) and A/quail/Shantou/2061/2000 (R-S-R-R), show growth curves in several mammalian cells and demonstrate the cleavage activation of the HA by serine proteases from human airway epithelium.

Key words: H9N2, hemagglutinin, proteolytic activation

P5

Cell tropism of pandemic H1N1 and H5N1 influenza A viruses in a human lung explant model

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Human influenza A viruses (IAV) and highly pathogenic avian IAV of the H5N1 subtype propagate efficiently in the human respiratory tract while low pathogenic avian IAV show low replication. Mechanisms controlling host susceptibility and cell tropism are widely unknown. In addition, most hypotheses are generated by using animal models and immortalized cell lines. Therefore, we established a lung explant infection model from authentic human patient material. In a first step we characterized the cellular tropism of different human and animal IAV and the distribution of virus receptors in the human lung by immuno- and lectinhistochemistry. Furthermore, we started to analyze the expression of potential antiviral factors.
In alveolar tissue, human IAV, highly pathogenic avian IAV of the H5N1 subtype as well as low pathogenic avian IAV, and classical swine IAV infected mainly type II alveolar cells whereas type I alveolar cells were not infected. In addition, all viruses could be shown to infect bronchial epithelial cells and less frequently alveolar macrophages. Interestingly, no differences regarding the cellular tropism could be found between the distinct viruses, which coincides with the detection of both, human and avian influenza virus receptors on alveolar and bronchial epithelial cells as well as on alveolar macrophages in this model.
Even though all tissue samples were infected with an equal amount of virus particles, counting of infected cells revealed that highly and low pathogenic avian IAV infected 4-6 times more cells per mm^2 of lung tissue compared to human and classical swine IAV.
In conclusion, the above described infection model provides an experimental approach with high clinical relevance to study IAV infection in the human lung.

Key words: cell tropism, receptors, human lung

P6

Development of gel agarose based conventional PCR assay for detection of pandemic H1N1 influenza virus (swine flu)

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Abstract: In April, 2009, pandemic H1N1 influenza virus known also swine flu H1N1 has caused the outbreak in Mexico, hence this led to a huge panic throughout the world. The concerned world authorities like WHO offered only real time solutions, therefore it was essential to develop an assay based on the conventional PCR method, where one can detect this strain in gel agarose, because conventional PCR equipments are easily available throughout the world, moreover they are much cheaper than the real time equipments and chemicals. Genekam developed an assay on the basis of the gene sequence (HA) from pandemic H1N1strain from Mexico after doing the alignment. A number of primers were developed. They were checking for their specificity in the blast and found out that a few of them are highly specific for swine flu H1N1. Moreover they were in the position to detect the strains from other areas like China, USA, Italy, Germany, Israel as well as other countries during the software analysis. The assay was developed as a two step assay i.e. in the first step, the isolated RNA was converted in cDNA with hexamer primers, dNTP etc. and in the 2^{nd} step, the cDNA were analysed with the specific primers (SG11 and SG12) for the presence of pandemic H1N1 signatures on gel agarose through electrophoresis. The other biggest advantage of this assay is that one can store the cDNA to do more analysis in the future while a disadvantage of this assay is that one needs more time against the one step real time PCR assay. Still this assay can be finished on the same day with widely available conventional PCR equipments, which can contribute to the monitoring as well as preventive process of outbreak. This assay was developed on California strain of swine flu. It was able to detect the different strains in the tests conducted in the laboratories in Mexico. There were no cross reactions with any related viruses like H5N1, seasonal H1N1, H3N2, H7N7 strains as well as healthy samples of human beings. This assay is highly sensitive, reproducible and a specific tool for the identification of human pandemic H1N1 with conventional PCR on gel agarose electrophoresis.

P7

Polymerase enhancing NP and PA mutations in the fatal human A/Hongkong/156/97 H5N1 isolate may provide a minor increment to virulence.

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Highly pathogenic avian influenza A viruses (HPAIV) are widespread in poultry and continue to pose a pandemic threat as they are occasionally transmitted to mammals including humans. During such events, these viruses undergo adaptation to mammalian host cells for efficient replication. Model studies on the mouse-adapted H7N7 HPAIV SC35M demonstrated that this adaptation was mediated by amino acid exchanges within the vRNP complex leading to enhanced polymerase activity in mammalian cells accompanied with increased virulence in mice (Gabriel et al., PNAS 2005). SC35M differs from its precursor SC35 by nine amino acid exchanges: seven of them within the polymerase subunits and the nucleoprotein. Interestingly, in the first human fatal H5N1 isolate A/Hong Kong/156/97 (HK156), two of these amino acids, NP-319K and PA-615R, were found in contrast to common avian and established mammalian strains.

To investigate the relevance of these exchanges, we established a plasmid-based reverse genetics system for HK156 and replaced those two amino acid residues by their avian counterparts. The resulting viruses were analyzed in-vitro for differences in replication and polymerase activity using a luciferase reporter assay in avian and mammalian cells. Furthermore, we investigated the impact of these mutations on virulence in mice. Substitution of PA R615K did not lead to remarkable differences in polymerase activity on HEK-293T cells (95% of reverse genetic wild type) or virulence in mice, as the LD_{50} of this mutant was the same as that of the wild-type (less than 1 pfu). In contrast, NP-319K is relevant for virulence to some extent, as this substitution impairs early replication in A549 cells, reduces polymerase activity to 80% and increases the LD_{50} in mice to approximately 4 pfu. Replacing both amino acids resulted in a slightly reduced polymerase activity (85%) and increased the LD_{50} to 2 pfu.

Taken together, the amino acids investigated in this study do not (PA-615R) or only to a very limited extent (NP-319K) increase virulence in mice. Therefore, other (additional) amino acids may contribute to severe disease following human H5N1 infections. Identification of amino acids contributing to high virulence of HPAIV in humans will enable an advanced risk assessment on the evolution of strains with pandemic potential.

Key words: H5N1, virulence, host change, polymerase

P8

Avian influenza viruses use different receptors for the infection of the respiratory epithelium in different avian host species

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Influenza viruses initiate infection by binding of the hemagglutinin to cell surface receptors containing sialic acid as a crucial determinant. The distribution of α-2,3 and α-2,6-linked sialic acids on different cell types and the viral preference for these receptors is believed to determine both the cell and species specificity of the virus.
We have characterized the infection by avian influenza virus strains of the H7 and H9 subtypes in tracheal organ cultures (TOCs) from chicken and turkey. TOCs preserve the natural arrangement of the epithelial cells. Infection by ciliostatic viruses, like influenza viruses, is easily detected by monitoring the ciliary activity.
To analyze the role of sialic acids in the onset of infection, TOCs were pretreated with neuraminidase to protect the cells from virus infection. As expected, enzymatic pretreatement retarded the ciliostatic effect of the H7 subtype virus. By contrast, no protective effect on the ciliostasis was observed in chicken TOCs infected by an H9N2 strain. However, infection of turkey TOCs by the H9N2 virus was neuraminidase-sensitive. This infection pattern was confirmed by immunostaining.
Fluorescent staining using specific lectins to visualize α-2,3 and α-2,6-linked sialic acids on the cell surface revealed that both chicken and turkey respiratory epithelial cells contain α-2,3-linked sialic acids; however, α-2,6-linked sialic acids were found only on the surface of the turkey but not chicken respiratory epithelium.
These findings suggest that avian influenza viruses use different receptors on their host cells depending on both the subtype of the hemagglutinin and the host species.

Key words: tracheal organ culture, hemagglutinin, sialic acids

P9

Dual function of p38 MAPK signaling in the primary and secondary host gene response to HPAIV infection

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Highly pathogenic avian influenza viruses (HPAIV) induce severe septic-hemorrhagic inflammation in poultry and men. There is still an ongoing threat that these viruses may acquire the capability to freely spread from human to human as novel pandemic virus strains that may cause major morbidity and mortality. One of the intriguing features of HPAIV infections is the induction of a cytokine burst that strongly contributes to viral pathogenicity. It has been suggested, that this cytokine overexpression is an intrinsic feature of infected cells and involves hyperinduction of p38 mitogen-activated protein kinase. Since HPAI viruses seem to have a strong tropism for the endothelium, these cells may contribute to viral pathogenicity. To address the role of p38 MAPK signaling in HPAIV infected endothelial and epithelial cells we performed global gene expression profiling experiments in the presence or absence of the p38-specific inhibitor SB 202190. We could show, that inhibition of p38 leads to reduced expression of interferons (IFN) and other cytokines after A/Thailand/1(KAN-1)/2004 (H5N1) and A/FPV/Bratislava/79 (H7N7) infection in both cell types. Furthermore, the expression of interferon stimulated genes, that were induced by IFN treatment or conditioned media from HPAIV infected cells was decreased when the target cells were preincubated with SB 202190. These observations show, that p38 acts on two levels of the antiviral IFN response: Initially the kinase regulates IFN induction and at a later stage p38 controls IFN signaling and thereby expression of IFN-stimulated genes.

Key words: p38 mitogen-activated protein kinase, highly pathogenic avian influenza virus

P10

Adaptation of an avian influenza A isolate to replicate in swine tracheal cells

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Introduction
Influenza lineages are believed to establish themselves in mammals through cross-species transmission from an avian reservoir followed by adaptation to the new host. This study focuses on viral adaptations allowing an avian viral isolate to adapt to better replication in swine cells.

Methods
A naïve avian influenza virus was adapted to continuous swine tracheal cells (NPTr) by repeated passaging. Adaptation, defined by improved viral growth in swine tracheal cells, was assessed by viral yield assay using real-time PCR. Genetic changes between the pre- vs. post-adapted virus were identified by direct sequencing of the full viral consensus genomes.

Results
Passaging resulted in a marked increase of viral yields in NPTr cells, on average by 1.5 $\log_{10}$ at one day post infection and 4.8 $\log_{10}$ at two days post infection. Furthermore, in a pilot assay, only the adapted virus seemed to grow in primary swine tracheal cells. Sequencing of the viral genomes identified nucleotide differences on segments 2, 3, 4, 7, and 8, and amino-acid differences on segments 2, 4 and 8. The parental field isolate was found to contain a sub-population of possibly two other influenza A viruses. Reassortment and spontaneous mutation contributed to the final constellation of genes that constituted the final pure swine-tropic virus.

Conclusion
This study suggests that amino-acid differences in segments 2, 4 or 8 may be important for avian influenza virus adaptation to pig cells, and that a combination of spontaneous mutation and reassortment during mixed infections can contribute to adaptation to a new host. Our current objective is to assess the relative importance of the various differences observed between the parental and the adapted viruses by generating defined reverse genetics clones and testing their infectivity in a range of pig models.

Key words: cross-species adaptation, emergence, avian influenza, swine influenza

P11

Avian influenza A virus monitoring in wild birds in Bavaria: occurrence and heterogeneity of H5 and N1 encoding genes

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Between January 2006 and December 2007 tracheal, cloacal swabs, or tissue samples (n=12,930) from various waterfowl species were collected and screened for AIV genome presence in Bavaria. In 291 (2.3%) birds genomes of influenza A viruses were detected by reverse transcription real–time PCR (RT-rPCR) targeting the matrix protein gene. Furthermore, solitary H5 hemagglutinin or N1 neuraminidase encoding genes were identified in 35 (0.3%) apparently healthy birds; whereas highly pathogenic (HPAI) H5N1 virus genomes were only diagnosed in dead wild birds (n = 93; 0.7%) found across this federal state region. In this study, multiple import events for H5N1 viruses were confirmed during 2006 and 2007. In addition, our findings argue against an existing HPAI H5N1 reservoir in aquatic birds in Bavaria. In contrast, phylogenetic analyses of the H5 or N1 sequences of low pathogenic avian influenza (LPAI) viruses revealed a marked diversity and multiple genetic lineages. This diversity of LPAI H5 and N1 subtype components indicates the existence of LPAI HA and NA gene pools which differ from the Bavarian HPAI H5N1. Moreover, the hemagglutinin amino acid differences between LPAI H5 viruses of a western European genotypic lineage observed in wild birds suggest a continuous evolution of LPAI viruses in Bavaria.

P12

0ligomeric organization of influenza A virus NS1 effector domain

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Influenza A viruses are important pathogens causing seasonal epidemics and occasional pandemics. The nonstructural protein NS1, encoded by segment 8 of the viral genome, is a 26-kDa multifunctional protein associated with various regulatory activities including both protein-protein and protein-RNA interactions, which can increase the pathogenicity and virulence of the virus during infection. NS1 has two domains to accomplish its multiple functions: the N-terminal RNA-binding domain can sequester the aberrant viral double-stranded RNA to antagonize the host's innate immune responses, and the C-terminal effector domain contains binding sites for several host-cell proteins. The X-ray structures of the NS1 effector domain from several virus strains have been determined, revealing the formation of a homodimer in the crystal. In spite of these structural studies, the precise oligomerization mode of NS1 effector domain remains unclear since different dimer configurations, e.g. a helix-helix dimer and a strand-strand dimer, were seen in the crystals. We focus on the NS1 effector domain from a highly virulent H5N1 strain (A/Vietnam/1203/2004) that was associated with disease outbreak in Thailand and Vietnam during late 2003 and early 2004 (case/fatality ratio ~60%). The protein was crystallized by the sitting-drop vapor-diffusion method and we solved its structure at 2.4 Å resolution. Different dimer forms of the H5N1 NS1 effector domain can be observed in our crystal structure as well, indicating the protein might form higher order oligomer than only dimer. Therefore, we mutated Trp182, which participates in the interface of the helix-helix dimer, to Ala and found that the mutant exists as a mixture of stand-stand dimer and monomer in solution (by small-angle X-ray scattering and size-exclusion chromatography). These results further indicate the oligomeric organization of the NS1 effector domain. Furthermore, the X-ray structure of a complex of NS1 effector domain (A//Udorn/72, H3N2) with the F2F3 domain of CPSF30 has also been determined by Das, K., et al. (*PNAS.* 2008. 105. 13092-7). Interestingly, two NS1 effector domains in this complex form a novel head-to-head dimer, which is unexpected and different from any published dimer structure of the NS1 effector domain alone. Therefore, the NS1 effector domain might need a conformational shift to interact with host cellular proteins. It may be that the NS1 effector domain has an oligomeric organization and dissociates to variable dimers or even monomer to fulfill its multiple functions of binding to different cellular interaction partners. To better elucidate the mode of action of NS1, the structure determination of the complexes between H5N1 NS1 effector domain and different host proteins is underway in our laboratory.

Key words: NS1 protein, crystal structure, oligomerization, host cellular proteins

P13

An increased host range of influenza A virus of subtype H5N1 is mediated by mutations PB2 S714I and S714R of the polymerase

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Background. Adaptation of the highly pathogenic avian influenza virus (HPAIV) SC35 (H7N7) to mice involves two important mutations, D701N and S714R, of the polymerase subunit PB2 that both increase the activity of the enzyme in mammalian cells (Gabriel et al., PNAS 102, 18590-18595, 2005). While mutation D701N has also been observed after adaptation of other influenza viruses to mammalian hosts, S714R was not found in any natural isolate. However, a cluster of viruses phylogenetically related to HPAIV A/Goose/Guangdong/1/96 (H5N1) have a similar mutation in PB2, S714I, parallelled by an increased host range. PB2 mutation D701N was not observed with the Gs/Gd-like viruses.

Methods. To understand, whether mutations PB2 S714I and S714R play a role in the adaptation of H5N1 viruses from avian to mammalian hosts and if there is an interdependence with mutation D701N, we performed polymerase activity assays with single and double mutants of A/Thailand/1(Kan-1)/04 (H5N1) polymerase (wildtype PB2 701N-714S). Furthermore, we studied growth kinetics of recombinant viruses bearing the respective single or double mutations in the PB2 subunit.

Results. In the mini-replicon assay, either substitution at aa714 (S714I or S714R) leads to a significant increase in activity of up to 10fold compared to wildtype polymerase. This effect can be observed in mammalian and avian cells and is independent of aa701. In human A549 cells there is also an increase in growth rates when mammalian-like rKan1-PB2 701N-714S (wildtype) and rKan1-PB2 701D-714R are compared to the avian-like mutant rKan1-PB2 701D-714S.

Conclusions. The finding that substitution of PB2 S714 to either 714I or 714R enhances viral polymerase activity suggests an adaptive potential. This is supported by the observation, that the mutant rKan1-PB2 701D-714R is able to grow better in human lung cells than rKan1-PB2 701D-714S.

P14

Pigs infected with pandemic influenza virus A/H1N1 lack cross reactive HI antibodies against Eurasian swine influenza viruses of subtype H1N1

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Experimental studies have shown that naive pigs are susceptible to pandemic influenza virus A/H1N1 and that the virus readily transmits between them (Lange et al., 2009; Brookes et al., 2010). Whether prior infection of pigs with endemic European swine influenza viruses (SIVs) may offer cross-protection against pandemic influenza virus A/H1N1 infection remained to be elucidated. Kyriakis et al. (2010) investigated the occurrence of serological cross-reactivity with pandemic influenza A/H1N1 after infection of pigs with European SIVs. Hemagglutination inhibiting (HI) antibodies were undetectable after a single infection with European SIVs but they were found in all dually, consecutively European SIV-infected pigs. These data suggest that pigs with infection-induced immunity to different European SIV subtypes may be at least partially immune against pandemic influenza A/H1N1. Broad serological cross-reactivity with pandemic influenza A/H1N1 in European pigs in the field further supports this notion (Dürrwald et al., 2010). In contrast to the previous studies we investigated the pandemic influenza virus A/H1N1 infection-induced cross-reactive immunity in pigs to 10 different endemic European SIVs of subtype H1N1 collected between 1979 and 2010. The results reveal that pigs infected with the pandemic influenza virus A/H1N1 do not develop cross-reactive HI antibodies to any of the Eurasian SIVs even when tested with hyper-immune sera, obtained from pigs re-immunised twice. Although these sera need to be further investigated for cross-reactive VN and NI antibodies, the results so far suggest that pigs infected with pandemic influenza A/H1N1 are not protected against infection with endemic European SIVs.

Key words: pandemic influenza virus A/H1N1, pigs, hemagglutination inhibitiontest, cross reactivity

P15

Novel preening-mediated transmission route of avian influenza viruses in aquatic birds

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Wild aquatic birds are reservoir hosts perpetuating the genetic pool of all influenza A viruses, including pandemic ones. High viral loads in feces of infected birds allow fecal-oral transmission. However, this route does not fully account for the efficiency of avian influenza virus (AIV) spread since dilution of infectious feces in water progressively decreases the chances of virus/host interaction. We investigated whether preen oil gland secretion, by which all aquatic birds make their feathers waterproof, could support a natural concentration mechanism of AIVs from water to birds' bodies, thus favouring virus spread and persistence in the aquatic environment. First, we detected consistently both viral genome and infectious AIVs on swabs taken by rubbing preened feathers of 345 wild mallards and examined by reverse transcription (RT)-PCR and virus isolation (VI) assays. Second, in two laboratory experiments using a quantitative real-time (qR) RT-PCR, we demonstrated that feather samples (n=5) and cotton swabs (n = 24) experimentally impregnated with preen oil, when soaked in AIV-contaminated waters, attracted and concentrated AIVs on their surfaces, as shown by statistical analysis. Third, we experimentally coated 7 mallards with a preen oil-AIV mix, and housed them with a control, uncoated, duck. Through self- and/or allopreening behaviour, all birds ingested the virus, as shown by virus detection in both oro-pharyngeal and cloacal samples. Virus isolation from cloacal swabs and virus-specific antibody response confirmed the occurrence of mallards' infection. Infectious AIVs were isolated from ducks' body surface until 32 days after the experimental coating.Our field and experimental results indicate that AIVs can be naturally concentrated and carried on the feather surface of infected ducks (i.e., those VI-positive from both cloacal and feathers swabs) and uninfected ones (i.e., those VI-positive from feathers only). In such a context, the natural preening behaviour, by which waterbirds spread preen oil all over their plumage (self-preening) or other birds' plumage (allo-preening), could facilitate the ingestion of AIV particles stuck on birds' feathers, thus promoting a preening-mediated infection route. Our findings also suggest that during the time period between the virus adhesion to the bird's body and the infection (possibly due to self- and/or allopreening), the virus could move in nature with the host by an undescribed circulation mechanism. We demonstrate here a novel viral transmission route that adds to, and possibly contributes to explain the knowledge of long-distance movements and long-term infectivity of lowly and highly pathogenic AIVs in nature.
Key words: Influenza, Preening, Feathers, Waterfowl.

P16

Comparative phenotyping of susceptibility/resistance patterns to murinized H1N1 influenza A virus in two inbred mouse lines, C57BL/6 and DBA/2

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Humans infected with influenza viruses show a variety of illnesses. The seasonal influenza is mostly a mild febrile syndrome but can also cause acute respiratory distress syndrome and bacterial pneumonia in immunocompromised people. According to the World Health Organization, 5-15% of the population is infected by the annual influenza epidemics, with worldwide 250 000 to 500 000 deaths every year.
The mouse has proven to be a valuable model to study influenza related illnesses. However, not all mice strains infected with influenza behave in the same manner. Among 7 inbred strains, coming from different ancestor lineages, we found different grades of resistance to a standardized infection with a murinized H1N1 influenza strain. DBA/2 proved to be the most susceptible, whereas C57BL/6 was the most resistant. This diversity could be explained by a difference in the innate and/or the adaptive immunity.
We have made an extensive phenotypic profile of both inbred mouse strains after infection. We showed significant differences in mortality, clinical score, pathological and histological alterations, cytokine response and the lung viral and neutrophilic load. These differences appear earlier and are more severe in DBA/2 mice. Already on day 3, we see a significant difference between both strains for the viral load, IL6 and the neutrophilic load.
From this, we concluded that the adaptive immunity does not seem to play an important role. Further experiments should precise if the distinct phenotypes observed in DBA/2 and C57BL/6 mice are due to differences in target cells susceptibility/permissivity and/or the innate immunity.

Key words: Influenza, H1N1, mouse, innate immunity

P17

ADAPTATION OF AVIAN INFLUENZA VIRUSES OF THE SUBTYPE H9N2 TO THE RESPIRATORY EPITHELIUM OF CHICKEN AND TURKEY

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We analyzed the adaptation of an egg-grown avian influenza virus of the H9N2 subtype (strain A/chicken/SaudiArabia/CP7/1998) to the respiratory epithelium of chicken and turkey. Tracheal organ cultures (TOC) served as a culture system which preserves the setting of epithelial cells as present in the avian trachea.
The parental virus was passaged four times in either chicken or turkey TOCs. The progress of infection was monitored by the ciliostatic effect induced by this virus. In the course of the passages, the virus underwent an adaptation process as indicated by a shorter time required for the induction of ciliostasis. The infection of chicken TOCs by the chicken TOC-adapted virus resulted in a complete ciliostasis three days earlier when compared to the egg-grown virus. Infection of turkey TOCs with the turkey TOC-adapted virus had a similar effect though it was less pronounced.
We will report the sequence changes in the hemagglutinin of the adapted viruses and their effect on the sialic acid binding activity of the viruses with respect to a preference for either α2,3 or α2,6-linked sialic acid.

Key words: adaptation, tracheal organ culture, sialic acids

P18

Influenza A virus infection causes specific changes to the nucleolar proteome including an NS1-dependent relocalisation of the RNA-editing enzyme ADAR1

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Influenza A virus (IAV) is a major human pathogen whose genotypic diversity results in unpredictable pandemics and epidemics. Interaction with the cell nucleus is essential to IAV infection, allowing recruitment of cellular components to facilitate virus replication. Viral proteins are also targeted to the nucleolus, a sub-nuclear structure involved in ribosomal biogenesis, RNA maturation, stress response and control of cell growth, but the functional consequences of this are unclear. We took an unbiased approach to studying IAV-nucleolar interactions by using stable isotope labeling with amino acids in cell culture (SILAC) in conjunction with LC-MS/MS to quantify changes in the nucleolar proteome following infection with A/PR/8/34 (H1N1) and A/Udorn/72 (H3N2) strains of the virus. Only a minority of nucleolar proteins showed significant changes in abundance after infection; these alterations were mostly different between the two strains but could be validated by confocal microscopy. Many of the affected proteins comprised functional groupings, including components of ribonuclease P, RNA polymerase I, the MLL1 histone methyltransferase complex, as well as nuclear paraspeckles and the adenosine deaminase acting on RNA (ADAR1) protein. Both long and short isoforms of ADAR1 relocalised to the nucleolus as early as 4 h post infection with both strains of virus. Infection with mutant viruses as well as plasmid transfection experiments showed that the dsRNA-binding domain of NS1 was both necessary and sufficient to cause this relocalisation. However, despite ADAR1's suspected role as an IFN-inducible anti-viral factor, initial results from siRNA depletion experiments suggest it acts as a positive factor during influenza infection. Overall, we conclude that IAV targets specific nucleolar pathways, potentially to benefit virus replication.

Key words: nucleolus, SILAC, ADAR1, SFPQ

P19

The polyphenol rich plant extract CYSTUS052 exerts potent antiviral activity against influenza- and rhinoviruses by preventing viral attachment to host cells

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Infections with influenza A viruses (IAV) still pose a major threat to humans and several animal species. The appearance of highly pathogenic avian H5N1 viruses and new H1N1v swine-origin influenza virus in humans as well as the increasing incidence of resistance to the currently available medication highlight the urgent need for novel antiviral drugs for prophylaxis and therapy. Here we demonstrate that the polyphenol rich plant extract CYSTUS052 from the Mediterranean plant *cistus incanus* exerts a potent anti-influenza virus activity in cells infected with various influenza viruses including those of the H5N1 and H1N1v type. The extract is also highly active against different types of human rhinoviruses (HRV). CYSTUS052 did not exhibit apparent harming effects on cell viability and did not influence metabolism, proliferation or cell activation by extracellular ligands. Furthermore, viruses did not develop resistance to CYSTUS052 upon consecutive passaging. Mechanistically, the protective effect appears to be due to a binding of the CYSTUS052-ingredients to the virus surface, preventing virus-binding to cellular receptors. Since these plant extracts are already in use in traditional medicine for centuries without reports of side effects, local application of CYSTUS052 to the respiratory tract may be a promising approach for prophylaxis and therapy of respiratory virus infections cause by IAV or HRV.

P20

The NF-κB-inhibitor SC75741 efficiently blocks influenza virus propagation by retention of the viral RNP complexes in the nucleus without the tendency to induce resistant virus variants

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Influenza is still one of the major plagues worldwide. The appearance of highly pathogenic avian H5N1 viruses or swine-origin H1N1v influenza viruses in humans and increasing incidence of resistance to the currently available medication highlight the need for new and amply available antiviral drugs. We and others have demonstrated that influenza virus misuses the cellular IKK/NF-κB signalling pathway for efficient replication suggesting that this module may be a suitable target for antiviral intervention. Here we show that the novel NF-κB inhibitor SC75741 efficiently blocks replication of influenza A and B viruses, including A/H5N1 isolates and H1N1v strains in concentration that do not affect cell viability or metabolism. The underlying molecular mechanism of SC75741 action involves impaired expression of proapoptotic factors, subsequent inhibition of caspase activation as well as block of caspase-mediated nuclear export of viral ribonucleoproteins (RNPs). Besides this direct antiviral effect the drug also suppresses virus-induced overproduction of cytokines and chemokines, suggesting that it might prevent the so-called cytokine burst that is an important pathogenicity determinant of infections with highly pathogenic influenza viruses, such as the A/H5N1 strains. Most importantly the drug did not shown any tendency to induce resistant virus variants. Thus, a SC75741-based drug may serve as a broadly active non-toxic anti-influenza agent.

P21

Influenza A virus activates the epidermal growth factor receptor (EGFR) kinase and generates a PI3K binding site in the EGFR for efficient entry into host cells

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Previous observations suggested that the entry of IAV is not constitutive but at least in part a signaling-regulated event. This implies the requirement for yet unknown signal transmitting receptors in addition to sialic acids which are already known as direct receptors for IAV attachment. We recently observed that the PI3K, an effector of growth factor receptors, is involved in IAV entry. Thus, we hypothesized that receptor tyrosine kinases (RTK) may play a role as cellular signaling receptors engaged by viral attachment to cells. Specific inhibition or knock-down of the EGFR, analysed as a model for RTKs, resulted in reduced viral uptake, in contrast to EGFR overexpression, demonstrating that EGFR expression is critical for efficient IAV uptake. IAV attachment, similar to EGF, led to a clustering of plasma membrane lipids and a redistribution and activation of EGFR resulting in the generation of a phosphorylated PI3K binding site in the EGFR. Using a sialic acid-specific lectin to mimic IAV binding, also resulted in the activation of EGFR. First indications suggest that p85 subunit of PI3K is recruited to the EGFR upon virus binding. Co-localization of IAV with lipid rafts (LR), in addition to the finding that disruption of LR impaired the entry of virions, suggests the requirement of LR for efficient IAV uptake. We propose that IAV is a multivalent agent that, upon binding to sialic acids, is able to cluster and activate EGFR leading to receptor-mediated signaling events, such as PI3K/Akt activation, which enhances IAV uptake.

P22

Pandemic influenza 2009 in Russia: isolation, antigenic analysis and biological properties of viruses

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Though sporadic cases of introduction of pandemic influenza 2009 A(H1N1)v were observed in Russia in May-July 2009, the first wave of pandemic started on 42nd week 2009 and lasted till 52nd week with the maximum isolation on week 47. During that period we isolated 417 pandemic strains from the different geographic regions of Russia, among them 35 strains from the post-mortem materials.

Isolation of the pandemic virus from nasopharyngeal swabs was equally effective in embyonated eggs and in the MDCK cells, though the strains isolated in eggs had higher hemagglutination titers and were more stable. Isolation from the post-mortem materials was effective only in eggs. Isolates of the first wave of pandemic 2009 in Russian Federation were antigenically homogenous and all isolates were similar to the reference strain A/California/07/09 (H1N1)v (reacted with the appropriate antiserum to 1/1-1/4 of homological titer, only 3 strains reacted up to 1/8). Russian pandemic isolates reacted also with the antiserum to the swine-origin influenza strain A/New Jersey/8/76 (1/2-1/8 of homological titer) and some of them – even with the antiserum to the strain A/Iowa/13/30 (up to 1/8 of homological titer). While more than 30 years had passed since the outbreak of "swine flu" in New Jersey the hemagglutinin of the modern pandemic H1N1 virus preserved some common antigenic determinants with old strains of swine origin.

The essential part of Russian pandemic isolates exhibited the ability to react with high titers (more than 1/1280) with heated at 56°-80° C equine serum while most swine viruses isolated earlier were inhibitor-resistant. In this respect all Russian strains form two distinct groups – A/California/07/09-like ("non-reactants") and A/St.Petersburg/56/09-like ("reactants"). For the pandemic strains isolated on the territory of Russia this property proved to be the most obvious criterion of the differentiation of their characteristics.

The wide spectrum of human and mammalian cell lines (10 lines tested) except MDCK proved to be practically non-sensitive to the pandemic A(H1N1)v isolates. The mutation in HA gene D222G was revealed in 9 of sequenced Russian strains. According to our data this mutation could not yet be attributed to any enhancement of the pathogenicity of the appropriate viruses. We also did not find any peculiarities of antigenic or other properties of strains isolated from the post-mortem materials.

In the end of epidemic season some influenza B viruses were isolated. During the current season neither epidemic A(H1N1) nor A(H3N2) viruses were isolated in our laboratory.

Key words: pandemic influenza 2009, virus isolation, hemagglutination-inhibitoin, thermostable inhibitors

P23

Antiviral activity of Influcid® in experimental model systems

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The complex preparation Influcid® (Deutsche Homöopathie-Union) is used in the prophylaxis and treatment of some acute respiratory diseases including influenza. The purpose of this work was to study its possible direct or indirect antiviral effects in cell cultures and in the model experimental influenza infection in mice.

In the *in vitro* studies we used a panel of etalon and/or epidemic influenza viruses of human and avian origin: A/H1N1 (epidemic), A/H1N1v (pandemic 2009), A/H3N2, B, A/H5H1 (avian, high- and low-pathogenic), H7N3, H9N2, herpes-virus (HSV-1 and HSV2) and adenovirus of the 3rd type. In mice the adapted to mice etalon strain A/PR/8/34 (H1N1) was used.

In vitro the preparation exhibited a moderate antiviral activity against most influenza viruses which was assessed in the hemagglutination test. Human viruses were as a rule inhibited more than avian. Much more pronounced was the activity of Influcid® assessed in MTT-test – a widely admitted index of cellular viability *in vitro*. The same effect was evident with adeno- and herpes-viruses.

We could conclude that the antiviral activity of Influcid® is to a considerable degree caused by a non-specific cytoprotective activity against the viral cytopathogenic effect.

In mice we could observe a statistically significant drop of mortality and some reduction of virus reproduction in the lungs when the preparation was daily administrated *per os* starting one day before the viral inoculation and for the three consecutive days after infection in pharmacologically adequate concentrations.

Key words: Influcid®, antiviral effect, influenza virus, herpes-virus, adenovirus, hemagglutination, MTT.

P24

Generation and Characterization of Monoclonal Antibodies to H5N1 Influenza Virus Using Recombinant Viral Peptides

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Background. More than 480 human cases of avian influenza caused by H5N1 influenza virus infection have occurred since 2003. Concerns about virus mutation, leading to facilitated human-to-human transmission warrant the development of rapid antigen detection. So far available non-molecular tests use the viral nucleoprotein and therefore do not distinguish subtypes.
Methods. Conserved H5-specific peptide sequences of the HA-protein (designated P1-P4) and chain HA1 (rHA1) were expressed in *Pichia pastoris* and used for generation of monoclonal antibodies (mAbs) by somatic cell hybridisation.
Results. Polypeptides rHA1, P1, P2 and P4 induced seroconversion in immunised mice. Sera analysis revealed peptide specific polyclonal antibodies which bind to native H5N1 virus. P1-, P2- and P4-specific sera reacted with influenza virus subtype H5 specifically. Six mAbs reactive with different sequences of the HA1 and HA2 moiety of H5N1 influenza virus (strain A/Thailand/1(KAN-1)/04) were obtained (IgG_1 and IgM subtypes) and further characterized in Western blot, Elisa, haemagglutination-inhibition assay, neutralisation assay and immune-fluorescence on virus-infected cell cultures. Fine characterisation of three mAbs directed against P1 and P2 was done and epitopes were determined. These are highly conserved within subtype H5 and not shared within subtype H1 and H3.
Conclusions. The obtained mAb will be used to develop a specific and sensitive test for rapid H5-antigen detection.

Key words: H5N1, monoclonal antibodies, recombinant peptides

P25

The effect of PB2 627 of Influenza A virus on viral replication and polymerase dynamics in the context of mammalian and avian cells.

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Influenza A virus displays host range specificity which makes adaptations necessary for avian strains to replicate efficiently in mammalian hosts. We studied the effect of the single amino acid mutation in the viral polymerase subunit PB2 at position 627 which is known to contribute to host restriction. We investigated the transcriptional ability and polymerase complex nuclear mobility in avian and mammalian cellular backgrounds in respect of this residue. We used PR8 as mammalian and H5N1/turkey/England/50-92 as avian PB2 proteins respectively in minireplicion assays, FRAP analyses, interaction studies as well as generating reverse genetics viruses to comparing viral growth properties.
We found that for the avian PB2, the identity of amino acid position 627 is of major importance for successful propagation in human cells. A 'humanising' lysine is required both for efficient viral transcription/replication as well as normal mobility of the polymerase in human cells, indicating differential interactions with host proteins that depend on that residue. However, in an avian cellular background there was little effect seen from altering position 627, both in transcription and mobility assays. Interestingly, while mutating the avian PB2 to a human 627K rescued the previously virtually nonexistent transcriptional activity to near PR8 wt levels, the reverse introduction of 627E into a PR8 background had a far less drastic effect in reducing polymerase activity than expected. The diffusional mobility on the other hand was similar to that of a fully avian PB2.
When PR8 reassortant viruses with different PB2 genes were examined, a stronger effect of 627E in a PR8 PB2 background was observed than in the minireplicon assay. While the PR8 627E and 627K viruses grew to similar titres in eggs, 627E growth was delayed in mammalian cells and the virus displayed a small plaque phenotype. Conversely, a virus with avian PB2 (with 627E) expressed readily detectable quantities of viral proteins in mammalian cells despite the near inactivity of the gene in minireplicon assays. This implies that the effect of the 627 residue cannot be assessed entirely accurately by plasmid-based assays and is suggestive of host-specific pleiotropic effects of the polymorphism.

P26

GETTING IT OUT: POTENTIAL ROLE OF INFLUENZA A VIRUS NON-STRUCTURAL PROTEIN 1 (NS1) IN VIRAL mRNA EXPORT

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Influenza viruses replicate and transcribe their genomic RNA in the nucleus of the host cell and therefore depend on nuclear host functions, such as mRNA splicing and export. In metazoans, transport of mRNA is tightly linked to splicing during which export factors are recruited to processed mRNAs. As most influenza virus mRNAs are not spliced, it is of interest to understand how viral mRNAs enter the export pathway and which factors are involved in this process. Recently, the NS1 protein that is known to antagonize the host interferon response was also shown to associate with viral mRNA, which led us to hypothesize that the NS1 protein promotes viral mRNA export.
We used a bimolecular fluorescence complementation (BiFC) assay that facilitates the detection of protein interactions *in situ* to screen for NS1 protein binding partners in the cellular mRNA export pathway. Furthermore, biochemical and mutational analyses were conducted to characterize the identified interactions. The BiFC screening revealed that the NS1 protein forms a complex with the mRNA export factors Aly and SF2/ASF. Both interactions were detected exclusively in the nucleoplasm of the cell. In addition, *in vitro* binding assays showed that purified NS1 protein binds directly to recombinant Aly and SF2/ASF, indicating a direct interaction in the host cell. Finally, we showed that viral mRNA was precipitated with NS1 and SF2/ASF. These findings suggest a model in which the NS1 protein acts as an adapter protein that links viral mRNA to the cellular export machinery by recruiting specialised host factors.

Key words: NS1, mRNA export, Aly, SF2/ASF

P27

Proteolytic Activation of Influenza A Viruses by Proteases from Porcine Airway Epithelium

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Influenza A viruses cause a severe infectious disease of the respiratory tract and are responsible for annual epidemics and occasional pandemics that affect millions of people worldwide. Cleavage of influenza A virus surface glycoprotein hemagglutinin (HA) by host cell proteases is essential for viral infectivity, highlighting HA-activating proteases as promising drug targets. Recently, three human proteases from human airway epithelium, the natural site of influenza infection, were identified that are able to cleave influenza HA with monobasic cleavage site: the trypsin-like serine proteases HAT, TMPRSS2 and TMPRSS4. Being a natural host of influenza A viruses, pigs are an important animal model system for influenza A viruses. As influenza viruses infect congenerous tissues and cause similar symptoms in humans and pigs, it can be assumed that the very same HA-activating mechanism is accomplished by homologous or very similar proteases in pigs. For this reason, the identification and characterization of homologous HA-activating proteases from porcine respiratory tract is of specific interest.
Here, we cloned a protease that is homologous to human serine protease TMPRSS2 (pTMPRSS2) from primary porcine airway epithelial cells. We found that recombinant pTMPRSS2 is expressed in different mammalian cell lines as a full-length (zymogen) and a mature form. Coexpression of HA and pTMPRSS2 in mammalian cells mediates cleavage of HA, and, moreover, MDCK cells expressing pTMPRSS2 support multicycle replication of influenza viruses. These data suggest that pTMPRSS2 is a candidate for proteolytic activation of influenza A viruses in porcine airway epithelium.

Key words: hemagglutinin, proteolytic activation, porcine airway epithelium

P28

Influenza Infection Causes an Inhibition of the Immune Response in Human Monocytes: the Role of the Rar-related Orphan Receptor alpha.

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Background: In contrast to patients infected with seasonal influenza viruses, patients suffering from an infection with highly pathogenic influenza viruses (HPAIV) like H5N1 show an overwhelming cytokine storm and systemic spreading of the infection. As monocytes are the main producers of cytokines and chemokines, we investigated their role in influenza infections and the development of the cytokine storm. Cell-type specific response patterns were pointed out by comparison with other cells of the innate immune system.
Materials and Methods: Primary human monocytes and macrophages as well as endothelial cells (HUVEC) were infected with three different influenza virus strains, the HPAIV strain A/Thailand/KAN-1/2004 (H5N1), A/FPV/79/Bratislava (H7N7) and A/Puerto-Rico/8/34. The cell response was analyzed by RT-PCR, FACS staining, Western Blot and Affymetrix gene array for three independent samples. Data obtained here were analyzed with the Expressionist Suite Software Package. For statistical analysis the student's t-test was used.
Results: RT-PCR experiments with human monocytes showed that the viruses induce a strong interferon response and that the cells release distinct cytokines and chemokines. Interestingly, H5N1 induced the most limited cell response which might be an escape strategy promoting systemic infection. These findings were confirmed in a genome-wide gene expression analysis. The data obtained here showed a clearly reduced inflammatory response in monocytes compared to macrophages and endothelial cells with the highest immunosuppression in H5N1 infected cells. All three viruses led to an inhibition of the nuclear factor kappa B (NF-κB), whereas an overrepresentation of the rar-related orphan receptor alpha (RORα), a repressor of inflammatory responses, was found. These findings could be varified by RT-PCR and Western Blot. Functional aspects and the biological relevance of an immunosuppression by RORα are validated by experiments with knockout-mice.
Conclusions: Clinical infection with H5N1 is associated with a cytokine storm. In contrast to this, human monocytes showed a reduced immune response after infection with H5N1 which could be related to differential recruitment of transcription factors. The reduced activation of monocytes by H5N1 could represent a critical escape strategy promoting systemic infection.

Key words: cytokine storm, monocytes, immunosuppression, rar-related orphan receptor alpha

P30

Application of the Real-Time PCR method for the detection of human Influenza A(H1N1) of Pandemic potential, in Albania.

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Backround: In March and early April a new-swine origin Influenza A(H1N1) virus (SOIV) emerged in Mexico and the US. The transmissibility of this virus spread worldwide to 30 countries becoming the first influenza virus with pandemic potential of the 21 century.
In this paper we introduce, for the first time the new technology of the Real-Time PCR in the diagnosis of Influenza. Here we show the high sensitivity of the method and the fast detection of the influenza virus using the 1-step TaqMan-based Real-Time Reverse Transcription PCR (rRT-PCR) assay.
Materials and Method: From June 2009 until May 2010 were collected 3167 clinical samples, suspected to contain the influenza virus, including N/NF swabs, and bronchoalveolar aspirates. The samples were provided from the Sentinel surveillance implemented throughout Albania. For the extraction of the viral ARN we used the commercial kit of QIAGEN. Meanwhile for the detection of the influenza virus RNA we used the 1-step (rRT-PCR) assay developed and provided along with primer/probe sets targeting the haemaglutinin genes, from WHO CC-CDC Atlanta Georgia, on a platform of ABI 7500 Real-Time PCR machine.
Results: We used this method to test 1956 clinical samples suspected to contain the pandemic virus. A total of 1045 samples were tested and confirmed near the Cantacuzino Institute.
Resulted positive to contain the novel pandemic virus A/H1N1 572 of them and also 11 positive cases died. Some positive samples by rRT-PCR, were isolated in MDCK cell line on BSL-2plus facility and the result showed a virus with low titers. As a WHO NIC part we sent to WHO-CC Mill Hill, some of the isolates for further phylogenetical analyses. They revealed that the pandemic strain circulating in Albania was the same with the one circulating in all Europe. They also screened the viruses for resistance to the sialidase inhibitors; they were all sensitive to both inhibitors.
Conclusion: Overall, the results revealed that, the rRT-PCR method is the most fastest and sensitive method for the specific detection of influenza, which implies an essential importance in the instigation of appropriate patient and public health management and also for disease surveillance.

Key words: rRT-PCR, Haemaglutinin, sialidase inhibitor.

P31

Crk adaptor protein expression is required for efficient replication of avian influenza A viruses and controls JNK mediated apoptotic responses.

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The non-structural protein 1 (A/NS1) of influenza A viruses (IAV) harbors several *src homology domain* (SH)-binding motifs which are required for interaction with cellular proteins, such as the p85beta subunit of PI3-kinase. A SH3-binding motif (aa 212-217 [PPLPPK]) within A/NS1 has been shown to be essential for binding to the cellular adaptor proteins Crk/CrkL. Both regulate diverse pathways in the cell including activation of the MAP kinase JNK, that was previously shown by us to mediate antiviral responses. To elucidate Crk/CrkL functions in infected cells we knocked-down expression of Crk/CrkL by a siRNA approach. We could demonstrate that only those IAV that encode an A/NS1-protein harboring the intact SH3-binding motif PPLPPK are attenuated upon downregulation of CrkI/II or CrkL, but not of CrkII alone. The PPLPPK site-harboring candidate strains could be discriminated from other strains by a pronounced viral activation of the JNK-ATF2 signaling module that was even further boosted upon knock-down of CrkI/II. Interestingly, this enhanced JNK activation did not alter type-I IFN-expression, but rather resulted in increased levels of virus-induced cell death and Caspase-9 cleavage. Our results imply that binding-capacity of A/NS1 to Crk/CrkL has evolved in virus strains that over-induce the antiviral acting JNK-ATF2 signaling-module and helps to suppress the detrimental apoptosis promoting action of this pathway.

Key words: A/NS1, CrkI/II and CrkL, JNK-ATF2, JNK-mediated apoptosis

P32

Experience with PCR diagnosis of pandemic (H1N1) 2009 influenza with focus on the type of specimens and possible PCR inhibition

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Introduction: Real-time PCR is a highly sensitive method for the detection of the viral nucleic acid (NA) from the isolate. At the very beginning of the 2009 A(H1N1) influenza pandemic, the sequence for the specific detection of the A(H1N1) virus had been published and this method became the gold standard for the virus detection. An internal control is used for test quality assurance and has proved helpful in the interpretation of results. Internal control positivity alone cannot be taken as a confirmation of a negative test result, since a variety of factors may cause interference with virus detection. Such factors are e.g. the timing of specimen collection from the onset of symptoms, viral load variability and viral propagation primarily in the airways of infected patients.

Material and methods:

Clinical specimens: nasopharyngeal swabs, bronchoalveolar lavage, lower respiratory tract (LRT) aspirate, sputum, pulmonary exudate, post mortem autopsy specimens and swabs from the lungs and trachea.

NA isolation: Micro-column (Invitek) and magnetic beads isolation (Roche), autopsy, sputum, LRT aspirate were homogenized with a MagNA lyser.

One-step PCR: CDC Real Time RT-PCR protocol for Detection and Characterization of Swine Influenza (2009).

Results:

A total of 800 nasopharyngeal swabs were analyzed with no PCR inhibition being observed.

PCR inhibition was found in specimens collected from the lower airways, or post mortem specimens. As total 276 of such samples was examined, 52 of bronchoalveolal lavage (7.7% inhibiting), 20 LTR aspirates (no inhibition), 19 sputa (15.8% inhibiting), 161 post mortem autopsy (24.2% inhibiting), 22 post mortem lung/trachea swabs (22,7 inhibiting).

Conclusion:

Nasopharyngeal swabs practically exclude the presence of PCR inhibitors. When analyzing specimens from the lower respiratory tract or post mortem material, the internal control is a must, as full or partial PCR inhibition can be frequently observed. If so a the decreased volume of NA isolate (from 5μl to 2-1μl) added to the reaction mixture can be helpful. According to our experience, in such a case, the inhibition only lasted in 3 materials (sputum and post mortem specimens).

The WHO recommended method uses human RNase P as the internal control whose amplification always indicates the presence of human tissues in the specimen but does not confirm the absence of PCR inhibitors. In samples with decreased Ct for internal control, the partial inhibition of PCR may occur. Importantly, a decreased volume of NA isolate in the reaction also lowers the detection limit.

Key words: RT-PCR, PCR inhibition, Pandemic (H1N1) 2009

P33

Genetic diversity of the 2009 pandemic influenza A(H1N1) viruses in Finland

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In Finland, the first infections caused by the 2009 pandemic influenza A(H1N1) virus were identified on May 10, 2009. During the next three months almost all infections were found from patients who had recently traveled abroad. In September 2009 the pandemic virus started to spread in the general population, leading to localized outbreaks and peak epidemic activity was reached during the weeks 43-48. The nucleotide sequences of the hemagglutinin (HA) and neuraminidase (NA) genes from viruses collected from 139 patients were determined. The analyzed viruses represented mild and severe infections and different geographic regions and time periods. Based on HA and NA gene sequences, the Finnish pandemic viruses clustered in four groups. Finnish epidemic viruses and A/California/07/2009 vaccine virus strain varied from 2-8 and 0-5 amino acids in HA and NA molecules, respectively, giving a respective maximal evolution speed of 1.3% and 0.9%. There was a clear correlation with the number of amino acid substitutions and time of sample collection. Based on 3-dimensional modeling of the HA and NA structures most amino acid changes in HA and NA molecules accumulated on the surface of the molecule and were partly located in antigenic sites. Three severe infections were detected with a mutation at HA residue 222, in two viruses with a change D222G, and in one virus D222Y. Also viruses with a change of D222E were identified. All Finnish pandemic viruses were sensitive to oseltamivir having the amino acid histidine at residue 275 of the neuraminidase molecule. The Finnish pandemic viruses were quite closely related to A/California/07/2009 vaccine virus. The nucleotide/amino acid changes within the HA or NA molecules were not clearly associated with increased epidemic potential or exceptionally high virulence. Thus, the viruses isolated from mild or lethal infections appeared to be very similar with one another and the severity of influenza disease is likely due to individual host factors. Continued laboratory-based surveillance of the 2009 pandemic influenza A(H1N1) is important in order to rapidly identify drug resistant viruses and/or virus variants with potential ability to cause severe forms of infection and an ability to circumvent vaccine-induced immunity.

Key words: pandemic influenza, evolution, 3-d modeling, virulence

P34

IMMUNIZATION WITH PLANT-EXPRESSED HEMAGGLUTININ PROTECTS CHICKENS FROM LETHAL HPAIV H5N1 CHALLENGE INFECTION

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Highly pathogenic avian influenza (HPAI) is a striking disease in susceptible poultry, which leads to severe economical losses. Inactivated vaccines are the most widely used vaccines in avian influenza virus (AIV) vaccination programs. However, these vaccines interfere with serological detection of wild type AIV infections in immunized populations. Using vaccines that allow differentiation between infected and vaccinated animals (DIVA strategy) would stop current stamping out policy. Therefore, novel vaccination strategies are needed to allow improved protection of animals and humans from HPAIV infection. The presented study analysed for the first time the immunogenic capacity of plant-expressed full-length hemagglutinin (rHA0) of HPAIV H5N1 in several vaccine formulations within the highly relevant host species, chicken. We were able to express plant-expressed rHA0 at high levels and could show that, when administered with potent adjuvants, it is highly immunogenic and can fully protect chicken against lethal challenge infection. Real-time RT-PCR and serological tests demonstrated only marginally increased virus replication in animals vaccinated with plant-derived rHA0 compared to animals immunized with an inactivated reference vaccine. In addition, use of plant-expressed rHA0 also allowed an easy serological differentiation of vaccinated from AIV-infected animals based on antibodies against influenza virus NP protein.

P35

SIMULATING ANTIGENIC FUTURE? *IN VITRO* IMMUNOESCAPE OF HIGHLY PATHOGENIC AVIAN INFLUENZA (HPAI) H5N1

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Partial immunity leads to antigenic drift by selectively favouring viral escape mutants that probably already exist in the quasispecies representing the ancestor virus. These escape mutants should comprise mutations that conserve or better increase antigenic changes in the surface glycoproteins. To investigate this phenomenon, HPAIV H5N1 was serially passaged 100 times in cell culture under the pressure of neutralising, polyclonal chicken-derived antisera. While a significant escape could be observed, it was not possible to generate viruses completely escaping from being neutralized by a high titer polyclonal antiserum. Full genome sequences of these mutants as well as of control viruses passaged without serum pressure were determined by next generation sequencing and analysed. Comparison of the sequence data revealed major changes especially in, but not limited to, the hemagglutinin of the escape mutants, allowing the prediction of possible cumulative sequence changes leading to escape from neutralizing immunity. However these changes were associated with the loss of virulence in chicken, but, the cleavage site of the hemagglutinin turned out to be unchanged defining these viruses still as potentially highly pathogenic.
Based on our data, we propose that escaping the neutralising antibody pressure can be predicted with an in vitro model, however it shifts the equilibrium in a quasispecies to reduced virulence and therefore the occurrence of escape mutants with a sufficient fitness in the field is a highly complex and probably cumbersome process for HPAIV.

P36

Potent inhibition of Influenza virus replication with novel siRNA-chimeric-ribozyme constructs

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A multitarget approach is needed for effective gene silencing for RNA viruses that combines more than one antiviral approach. Towards this end, we designed a wild-type (wt) chimeric construct, that con- sisted of small hairpin siRNA joined by a short intracellular cleavable linker to a known, hammerhead ribozyme (Rz), both targeted against M1 genome segment of influenza A virus. When this, wt chimeric RNA construct was introduced into a mammalian cell line, along with the M1 substrate, encoding DNA, very significant (67%) intracellular down regulation in the levels of target RNA was, observed. When the siRNA portion of this chimeric construct was mutated keeping the Rz region, unchanged, it caused only 33% intracellular reduction. On the contrary, when only the Rz was made, catalytically inactive, keeping the siRNA component unchanged, about 20% reduction in the target M1, specific RNA was observed. This wt chimeric construct showed impressive (>80%) protection against, virus challenge, on the other hand, the selectively disabled mutant constructs were less effective. Thus, in this proof of concept study we show that varying levels of protection against virus challenge was, observed with novel mutant versions of the chimeric constructs.

P37

New Generation of Influenza Rapid Tests[i]

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Key words: influenza virus, POCT, sialoglycoconjugates, diagnosis

Rapid diagnosis of influenza viruses is important for the initiation of antiviral therapies, judicious use of antibiotics, hospitalization of patients and identifying local epidemics in a timely manner (Noyola et al., 2002).
Compared to real-time-polymerase chain reaction (RT-PCR) assays the rapid point of care tests (POCT) are less time - consuming and don't require skilled personal. However, they are not as sensitive as RT-PCR and therefore difficult to use for therapy or epidemiology relevant questions.
Most of the POCT available on the market uses a lateral flow immunoassay directed to influenza type A and/or type B antigens (nucleoproteins) directly from respiratory samples. The new type of influenza rapid POCT uses two surface glycoproteins of the influenza virus, namely hemagglutinin (HA) and neuraminidase (NA), for sensitive and selective detection. These two proteins play an important role in the infection process; HA is responsible for the binding of the virus to the host cells and NA is involved in the replication process and responsible for the release of virus progeny.
The POCT principle is based on sialoglyco receptor structures which bind selectively the influenza virus via HA (Gambaryan et al., 1997). The detection is directed to the NA. Fluorescent labelled active probes act as a "suicide substrate" and form a covalent linkage with the NA (Lu et al., 2005). This multiple fluorescent labelling of the virus allows a sensitive detection.
Depending on the host specificity or pathogenicity of the certain influenza virus it will be recognized by different sialoglyco receptor structures. Avian influenza viruses show high affinity to Neu5Acα(2-3)Gal-terminated oligosaccharides where as human influenza viruses show high affinity to Neu5Acα(2-6)Gal-terminated oligosaccharides. Changing the inner part of the oligosaccharide receptor structure high and low pathogenic isolates can be distinguished (Gambaryan et al., 2005 and 2006).

The poster will show the principle of the new influenza POCT and the first results. The mentioned sialoglyco receptor structures and the suicide-substrate have been synthesized and bound to nitrocellulose or to fluorescent latex beads, respectively. A lateral flow assay has been built up, optimized and tested with different influenza viruses.
In conclusion, the first results are looking promising for a new selective and sensitive influenza pandemic POCT. The test can be performed in less than 15 min and the results can be confirmed by a RT-PCR directly from the test strip.

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P38

REASSORTMENT BETWEEN AVIAN AND HUMAN H3 INFLUENZA STRAINS: WHICH SEGMENTS CO-SEGREGATE WITH THE AVIAN VIRUS HEMAGGLUTININ?

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Reassortment of influenza A viruses is the underlying mechanism of antigenic shift which led to several pandemics in the past. However, the molecular compatibility of the hemagglutinin (HA) gene from avian viruses with the genes of a human acceptor virus has remained unclear. Here, we studied which genes of an avian strain co-segregate with its HA gene after double-infection with a human strain under forced selection for the heterologous HA. For that, we generated a strictly elastase-dependent HA cleavage-site mutant from A/Hongkong/1/68 (H3N2) (Hk68) via reverse genetics and performed co-infections of A549 cells with the avian strain A/Duck/Ukraine/1/63 (H3N8) (DkUkr63) in absence of elastase but presence of trypsin in order to isolate reassortants carrying the avian virus HA exclusively. From plaque-purified reassortants, their segment composition, plaque morphology, and growth curves were determined. Genotyping of 21 plaques revealed that 16 were reassortants carrying the avian HA - either alone or in combination with other avian segments. Among these reassortants, the half contained the avian NA segment and formed large plaques like DkUkr63. Remarkably, sometimes the DkUkr63 PB1 and NP segregated together with the DkUkr63 HA whereas PB2 and PA always originated from Hk68. We conclude therefore that A/Duck/Ukraine/1/63 and A/HongKong/1/68 easily form DkUkr63-HA-carrying reassortants with different gene constellations including those of pandemic viruses with the avian virus NA and/or PB1 segments. Generally, an elastase-dependent HA cleavage site mutant of a human strain as acceptor model allows to assess the molecular correlates of gene segment compatibility and, beyond that, the ability of an avian strain to donate its HA segment to circulating human strains resulting in novel, antigenically heterologous reassortants with pandemic potential.

Key words: pandemic, avian influenza, reassortment, antigenic shift

P39

Prevalence of antibodies against Eurasian swine influenza viruses in humans with occupational exposure to pigs

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The Eurasian lineages of swine influenza viruses differ genetically from classical swine H1N1 influenza viruses and comprise avian-like H1N1, and human-like H1N2 or H3N2 subtypes. Although sporadic isolation of such viruses from human specimens has been reported, the prevalence of human infections is not known. Thus, the seroprevalence against Eurasian swine influenza viruses was investigated in two studies.
In a pilot study, sera were collected in Thuringia, Germany, from December 2007 to April 2009. This study group comprised 118 professionals with occupational exposure to pigs (50 pig slaughterers/meat inspectors, 46 pig farmers, 22 veterinarians caring for pig herds). The control group included 118 age- and gender-matched blood donors from Thuringia. As a result, 18 sera of the study group were identified with raised hemagglutination-inhibition titers against a panel of nine swine influenza viruses (three strains/subtype). For 17/18 sera this finding was confirmed in the neutralisation assay. For 11/18 sera the raise of titers was significant, i.e., a fourfold increase of hemagglutination-inhibition titers was observed. No gender-specific bias of the high titer sera was observed. Twelve sera of the control group showed increased hemagglutination-inhibition titers against swine influenza viruses. Hemagglutination-inhibition titers of 2/12 control sera were raised fourfold but did not exhibit a significant increase of neutralisation titers. All increased hemagglutination-inhibition titers of the control group may be explained by cross-reactivity with seasonal influenza virus strains, as all these sera also reacted with human strains.
Within the exposed group seroprevalences were determined with 1.7 % (0.0 – 6.0; 95% CI) for swH1N1, 5.9 % (2.4 – 11.9; 95% CI) for swH1N2 and 13.6 % (7.9 -21.1; 95% CI) for swH3N2 (see also Krumbholz et al., J Med Virol, 2010).
The findings of the pilot study were largely confirmed by preliminary data of a follow-up serosurvey among attendants of a German Veterinarians Conference.
In conclusion, both studies clearly demonstrate the zoonotic potential of Eurasian swine influenza viruses.

Key words: zoonotic infection, swine, human, seroprevalence

P40

Identification of Lethal Combinations of Packaging Signal Mutations Suggests an Assembly Checkpoint at the Level of the Genome During Influenza A Virus Morphogenesis

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It is generally accepted that influenza A virions usually incorporate eight separate RNPs containing one copy of each of the genomic segments, and that this is achieved through the operation of *cis*-acting segment-specific packaging signals located in the terminal regions of the vRNAs. The mechanism by which these signals function is uncertain, but studies have shown that their mutation can lead to drops in virus replication of ~ 10 – 1000 fold.

Surprisingly however, we found that combinations of packaging mutations in two separate segments often exhibited a synergistic effect on virus fitness, to the point that certain combinations were unable to be rescued by reverse genetics, suggesting a lethal phenotype. A systematic analysis of pairwise combinations of packaging mutations in any two segments showed that most, but not all double mutants caused a synergistic reduction of over 10,000-fold in virus growth. Since purely random packaging of any eight RNPs would be predicted to produce around 1 in 400 infectious particles, we interpret this as suggesting that this mechanism is not available as a fall-back option after disruption of specific packaging and that therefore a checkpoint likely exists to prevent assembly of virions with grossly defective genomes.

To test these hypotheses, we are currently studying a virus containing lesions to the segment 6 and 7 packaging signals that grows sufficiently well in eggs to permit experimental analysis but is nevertheless attenuated by over 100,000-fold in MDCK cells. Initial analysis of vRNA trafficking in cells infected with this virus suggests that the block to efficient virus replication occurs after nuclear export of the genome. Moreover, it has been possible to isolate revertant viruses from serial passage that maintain the initial lesions. Identification of the suppressor mutations is likely to shed further light on the packaging mechanism of influenza A virus.

Key words: packaging

P41

Matrix epitope coupled to HIV Tat protein transduction domain elicits CTLs to counter the influenza virus infection in mice

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Influenza viruses continue to pose a severe threat worldwide, causing thousands of deaths and enormous socio-economic loss. The major problem in fighting influenza virus is the high genetic variability which allows the virus to infect new host species and quickly overcome protective immunity. Vaccination with viral epitope, conserved in all the types and sub-types of influenza A virus, may efficiently counter the virus infection. The study was carried out in which the protein transduction domain (PTD) of Tat protein of human immunodeficiency virus (HIV) was fused to the epitopic segment of the matrix gene of influenza A virus as the PTD domain is known to deliver the peptides $\leq$ 10 kDa into the eukaryotic cells without the assistance of any external agent. The ligated oligo was cloned in pSecTag2 vector and expressed in MDCK cell line. The fusion protein was isolated, purified and expressed on antigen presenting cells (APCs) to generate immune response against the virus infected cells. The sensitized dendritic cells (DCs), when co-incubated with the cultured naive T cells, generated the cytotoxic T lymphocytes (CTLs) against the virus infected cells. The *in vivo* studies revealed the marked reduction in plaque count in lungs of Balb/c mice when the intra-peritoneal (i.p.) injection of sensitized DCs was followed by intranasal instillation of influenza A virus strain [A/PR/8/34 (H1N1)]. The real time RT PCR assay and western blot analysis also showed a significant decrease in viral titer as compared to the mock infected mouse. Cytokine analysis of the BAL fluid collected from these mice showed the enhanced expression of cytokines viz. IFN γ, IL-6, IL-5 and IL-4. Thus, this strategy represents a versatile system as a single carrier protein could be easily and rapidly coupled to the desired peptide to sensitize the APCs and thus activate the host immune system against the pathogenic virus.

Key words: Influenza, Antigen Presenting Cells, Cytotoxic T Lymphocytes

P42

Efficacy of a recombinant NDV-H5 vaccine against an Asian Highly Pathogenic clade 1 H5N1 virus in chickens with or without maternal-derived-antibodies.

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Avian Influenza viruses are enveloped RNA-viruses belonging to the Orthomyxoviridae family. The highly pathogenic H5N1virus can cause severe, contagious and fatal disease in poultry resulting in high economic losses in the poultry industry around the world. Since its emergence in 1997, the Asian HPAI H5N1 virus spread throughout South-East Asia to Europe, Africa and Middle East. In Europe, the presence of the Asian HPAI H5N1 viruses remains local and sporadic. In order to control the H5N1 virus infections in poultry, efficient vaccination strategies are strongly desirable.

In this study, the vaccinal potency of a recombinant NDV-H5 vaccine against the Asian clade 1 HPAI A/crested_eagle/ Belgium/01/ 2004 H5N1 strain was evaluated. Different vaccination strategies were tested in SPF chickens and in SPF chickens with maternal-derived-antibodies (MDA).

First, different routes of vaccination (drinking water, oculonasal and combination of both methods) were tested and showed that the vaccination by drinking water gives the best protection. Besides, it was demonstrated that the protective efficacy of the recombinant vaccine NDV-H5 increased with the dose in SPF chickens. The highest dose ($10^7 EID_{50}$/dose) of vaccine protected SPF chickens against the mortality, clinical symptoms and viral excretion against the Asian clade 1 HPAI H5N1 challenge strain. Anyway, some interference with the protection induced by the vaccine was detected in SPF chickens with H5- and NDV-MDA. However, in presence of NDV-MDA, the protection was better than in the SPF chickens with both NDV- and H5-MDA. All together, these results tend to prove that the recombinant NDV-H5 vaccine represents a potential candidate vaccine against the Asian clade 1 HPAI H5N1.

Further studies will be performed to investigate the range of protection against HPAI H5 viruses that can be obtained with this recombinant NDV-H5 vaccine. Additionally, the bivalence of the vaccine will be evaluated by challenge experiments with velogenic NDV strains.

P43

Molecular and physiological characterization of a novel, micro-RNA-based immune evasion mechanism of influenza A virus

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Recently, our group discovered a novel immune evasion strategy of influenza A virus (IAV) (unpublished data). We found that IAV infection of A549 human lung epithelial cells *in vitro* leads to a strong up-regulation of a specific cellular microRNA (miRNA), and that this causes post-transcriptional silencing of an interferon-induced antiviral protein and enhanced viral replication. In the future, we aim to characterize this immune escape mechanism in more detail, both on the molecular and the physiological level. We will answer the following questions: (A) What are the molecular mechanisms underlying the IAV-induced miRNA up-regulation? (B) Does this mechanism have an *in vivo* relevance?

To understand the molecular mechanisms responsible for the IAV infection-caused induction of the miRNA of interest, we will identify both the viral structure or process inducing the miRNA as well as the cellular signal chain required for this. In order to find the exact trigger for up-regulation of the miRNA during IAV infection, we will treat A549 cells with isolated viral components and examine the expression of the miRNA by quantitative real-time PCR. For the investigation of the signaling machinery involved in IAV-driven expression of the miRNA of interest, we will first screen for the transcription factor activating the miRNA's expression and subsequently analyze the upstream signal pathway biochemically.

In order to characterize the physiological relevance of the miRNA-mediated IAV immune escape strategy more precisely, we will develop a mouse model. To this end, we will investigate by cell biological and biochemical means if the miRNA is able to silence the murine homolog of the targeted antiviral protein, *in vitro*. If this is the case, we will analyze if this process occurs also *in vivo* and, by using inhibitors for the miRNA of interest as well as generating a miRNA knock-out mouse, how it affects the pathogenesis of IAV infection.

The results which we will generate in the future will help to understand the interactions between IAV and its host in more detail and may reveal novel options for antiviral therapy.

Key words: Immune evasion; micro-RNA; interferon-inducible antiviral gene; mouse model

P44

Phylogenetic predictions to trace host adaptation of H2N2 influenza viruses

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Besides causing yearly epidemics, influenza A viruses have the potential to seed pandemics. Reassorted viruses comprising gene segments of different species can infect naïve hosts but need to adapt subsequently to spread efficiently. In the case of 1957 "Asian" influenza A virus the HA, NA, and PB1 gene segments were of avian origin. Exactly which adaptive changes occurred in the 1957 influenza A virus remains unknown.

We hypothesize that ancestral sequences at the inner nodes linking the avian with the human cluster in a phylogenetic tree reflect the adaptation process of influenza A viruses after species transmission. These ancestral viruses are similar to those that most likely initiated the pandemic.

An alignment of human and avian influenza A virus segment sequences was generated. Phylogenetic trees based on Maximum Likelihood algorithms were calculated (PAUP*). This data was used to predict nucleotide sequences at the inner nodes of the tree (Paml). Only substitutions at the amino acid level were taken into consideration. The correct positioning of the sequences at the inner nodes was confirmed in a phylogenetic tree using the translated amino acid sequences (Phyml). The predicted mutations were introduced both into a human and an avian influenza A prototype virus segment resulting in gene segments that are identical at the amino acid but differ at the nucleotide level for the three genes of interest. Infectious viruses were recovered using reverse genetics techniques. Reassortant viruses were generated using an avian, a human prepandemic and a pandemic virus backbone.

We were able to determine ancestral nucleotide sequences located at the nodes connecting avian and human virus sequences in phylogenetic trees. 8 mutations in HA, 15 mutations in NA and 3 mutations in PB1 between the common ancestor and the closest avian and human H2N2 prototype viruses were identified. All predicted mutations were introduced in genes of viruses from the human and avian clade. Recombinant viruses containing 1, 2 or 3 ancestral gene segments were rescued. For PB1 single reassortant viruses no significant differences in replication pattern were observed *in vitro*.

The above-described method offers the possibility to calculate hypothetical common ancestors of viral genes that do not longer exist. These recreated ancestral viruses link avian and human wildtype viruses and though hypothetical they genotypically closely resemble the influenza A virus that probably seeded the 1957 pandemic. *In vitro* and *in vivo* studies will elucidate if these viruses have an intermediate phenotype as well.

Key words: phylogeny, ancestry, pandemic, H2N2

P45

Development of new anti-flu compounds directed against the interactions between the PA and PB1 subunits of influenza A virus RNA polymerase

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Influenza A virus causes an airborne highly-infectious disease, characterized by high morbidity and significant mortality. To date, anti-flu drugs are available, but they suffer from important drawbacks. Moreover, although an anti-flu vaccine is available, it has to be changed every year because the virus is prone to antigenic modifications. Thus, there is an urgent need for novel therapeutic interventions against influenza A virus, highlighted by the ongoing pandemic of a novel H1N1 swine-derived virus as well as significant zoonotic transmission of highly pathogenic H5N1 viruses.
A possible novel anti-influenza strategy is represented by the disruption of the protein-protein interactions among the subunits of the viral polymerase complex which consists of three proteins (PB1, PB2, and PA). The influenza virus polymerase is conserved among different viral strains and an analysis of the interactions between PA and PB1 recently showed that relatively few residues drive the binding, suggesting that the inhibition of such an interaction by small molecules could be a feasible strategy.
From an *in silico* screening of ~ 10 millions of virtual compounds, 32 potentially active molecules were selected. These compounds were tested for their ability to disrupt the physical interaction between PA and PB1 by an ELISA. By this assay, we have identified a few compounds with an IC_{50} comparable to that of a peptide corresponding to aa 1-25 of PB1, which represents the region of interaction with PA and was previously shown to inhibit PA-PB1 binding. Moreover, the cytotoxicity of the active compounds was evaluated in some cell lines (293T and MDCK). The effects of the compounds on flu polymerase activity and influenza virus replication were also investigated by mini-replicon assays and plaque reduction assays, respectively. In addition, analogs of the active compounds have been developed and characterized.

Key words: RNA polymerase, PA-PB1 interaction, in silico screening, new antivirals

P46

Host cell adaption of influenza A virus H5N1 by mutations in the distal domain of influenza virus hemagglutinin

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ABSTRACT Avian H5N1 influenza A virus has recently spread to numerous countries in Asia, Africa and Europe causing substantial mortality in poultry flocks and wild waterfowl. Infection with the virus is mediated by the influenza spike protein hemagglutinin (HA) which is recognized by sialic host cell receptors enabling uptake of the virus particle by endocytosis. In the acidic environment of the endosome the HA undergoes an irreversible conformational change triggering fusion of the viral envelope with the endosomal membrane and releasing the viral genome into the cytoplasm.
HA is organized as a noncovalent-associated homotrimer, each monomer being post-translationally cleaved into disulfide-linked HA1 and HA2. Mutagenesis of HA of virus strain X31 (H3 subtype) showed that electrostatic interactions - in particular salt bridges bridges between the two subunits of a monomer (intramonomer) and between monomers (intermonomer) - play an essential role for the pH-dependent stability of the HA ectodomain.
Disruption of a highly conserved salt bridge between monomers in the distal domain of HA significantly reduced its stability at low pH at the same time enhancing its fusion potential. Such mutations were found in human pathogenic H5N1 virus strains, putting forward the hypothesis that reduced stability of the HA ectodomain at low pH increases its infectivity for humans. However, further analysis is still necessary to prove the relevance of salt bridges, and thus of HA stability, for the adaption of the influenza A virus H5N1 to the human host.

Key words: influenza virus hemagglutinin, salt bridges, pH stability, host cell adaption

P47

Chasing notifiable avian influenza (NAI) in domestic poultry: a case report of low pathogenic avian influenza (LPAI) H5 viruses in two Belgian holdings.

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At the end of December 2008, two geographically distant holdings were found H5 positive during the annual AI serological screening in Belgium. Following these positive results, cloacal swabs were sampled for virological tests and a low pathogenic avian influenza (LPAI) virus of subtype H5N2 was isolated in the first holding and identified by Real Time PCR in the second holding. The first farm was a mixed holding with mixed ornamental birds and poultry and the second one was a free range breeding geese farm (about 1500). There were neither clinical symptoms nor mortality. Control measures were taken as foreseen in Council Directive 2005/94/EC with notification in ADNS and to OIE. A stamping out of ducks, geese, chickens, pheasants and turkeys was performed whereas the other birds were isolated and tested again after 1 month. Fifty animals were sampled during the stamping out: 10 were positive in serology for H5 and 12 were positive in RRT-PCR. The quarantined birds were still negative after one month and relaxed. Partial sequencing of the hemagglutinin gene of the H5N2 isolated showed a close homology with some recent H5N2 LPAI isolated in Germany.
It is noteworthy that, during the previous years, these two holdings were already found H5 positive by HI tests and swabs were collected but no virus could be detected. To have a better understanding of the "silent" circulation of the H5N2 isolate in the first holding, experimental infections on chickens and turkey were performed. They indicated that the H5N2 LPAI isolated was not fully adapted to this two poultry species, which might partly explained the persistence of the virus with no important burst in the holding.
In addition, some years, sera of the two holdings were found positive only with the first line H5 antigen and, as consequence, the flocks were declared as negative several times. It must be noticed that neither clinical symptoms nor mortality were registered during the whole survey. Our results indicate several limitations in the serological screening for early detection of LPAI and are a strong indication for increased virological monitoring in at risk farms.

Key words: serological screening, notifiable avian influenza, H5, diagnostic

P48

Identification and Characterization of Interferon-producing Cells in Chicken

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Highly pathogenic avian influenza viruses (HPAIV) of the subtype H5N1 have their natural reservoir in migratory waterfowl. These viruses have the ability to adapt rapidly to gallinaceous birds and cause severe disease with extremely high mortality rates of up to 100\%. It was shown before that chickens infected with H5N1 viruses exhibit extraordinary high levels of type I Interferon (IFN), despite the fact that theses viruses have a functional NS1 protein (non structural protein 1) which normally inhibits IFN production. So far it was not clear why IFN is induced in such high amounts, where it comes from and why it fails to protect chickens from HPAI viruses. This study focuses on the question what the source of the type I IFN is. It was hypothesized that there is a special IFN-producing cell in chickens which has similar properties as the mammalian plasmacytoid dendritic cell (pDC). To test this hypothesis, staining for IFN was performed on lymphoid tissue of HPAIV-infected chickens. It was possible to detect IFN-producing cells in the spleen and cecal tonsils by staining of cryosections. Furthermore it was shown that these cells do not need to be productively infected in order to produce large amounts of IFN, even though infection of them occurs in rare cases. Moreover, in chickens treated with imiquimod, a toll-like receptor 7 (TLR7) agonist, IFN-producing cells were also detected in the spleen. Whether these are the same cells as in HPAIV-infected chickens is not clear yet. To gain further insight into the nature of these cells, different cell markers were used to identify them. The pan leukocyte marker CD45 seems to be expressed at rather low levels on all IFN-producing cells, while markers for Bcells and myeloid cells were expressed very heterogeneously. Remarkably, staining pattern of cell markers were similar in HPAIV-infected compared to imiquimod-stimulated chickens. In conclusion these data indicates that there might be a pDC-like cell in chickens. These cells can be stimulated with TLR7 agonists like imiquimod and upon infection of chickens with HPAIV. How these cells are stimulated after HPAIV infection stays unclear. It seems that NS1 is in principle able to block IFN induction but fails to do so in these IFN-producing cells. IFN-producing cells seem to belong to the leukocyte lineage but it remains unclear if they form a distinct sublineage or are a heterogenous cell population.

Key words: IFN-producing cells, chicken, H5N1, Imiquimod

P49

Influence of glycyrrhizin on H5N1 replication and pro-inflammatory gene expression in human macrophages and lung cells

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Hypercytokinaemia is thought to contribute to highly pathogenic H5N1 influenza A virus disease. Glycyrrhizin is known to exert antiviral, immunomodulatory, and anti-inflammatory effects. Here, the effects of an approved parenteral glycyrrhizin preparation were investigated on highly pathogenic influenza A H5N1 virus replication, H5N1-induced apoptosis, and H5N1-induced pro-inflammatory responses in lung epithelial (A549) cells. Therapeutic glycyrrhizin concentrations substantially inhibited H5N1-induced expression of the pro-inflammatory molecules CXCL10, interleukin 6, CCL2, and CCL5 (effective glycyrrhizin concentrations 25 to 50 µg/ml) but interfered with H5N1 replication and H5N1-induced apoptosis to a lesser extent (effective glycyrrhizin concentrations 100 µg/ml or higher). Glycyrrhizin also diminished monocyte migration towards supernatants of H5N1-infected A549 cells. The mechanism by which glycyrrhizin interferes with H5N1 replication and H5N1-induced pro-inflammatory gene expression includes inhibition of H5N1-induced formation of reactive oxygen species and (in turn) reduced activation of NFκB, JNK, and p38, redox-sensitive signalling events known to be relevant for influenza A virus replication. In human monocyte-derived macrophages (MDMs), glycyrrhizin 100 µg/ml, a therapeutically achievable concentration, impaired H5N1-induced production of CXCL10, interleukin 6, and CCL5 and inhibited H5N1-induced apoptosis but did not interfere with H5N1 replication. Although control of hypercytokinaemia is considered to be a therapeutical aim within anti-influenza therapies, global inhibition of immune responses may result in the loss of control of virus replication by cytotoxic immune cells including natural killer cells and cytotoxic $CD8^+$ T-lymphocytes. Notably, glycyrrhizin concentrations that inhibited H5N1-induced pro-inflammatory gene expression did not affect cytolytic activity of natural killer cells. In conclusion, glycyrrhizin-mediated effects on H5N1 replication are cell type-dependent while therapeutic glycyrrhizin concentrations inhibit H5N1-induced pro-inflammatory gene expression in human macrophages and lung cells. Since H5N1-induced hypercytokinaemia is considered to play an important role within H5N1 pathogenesis, glycyrrhizin may complement the arsenal of potential drugs for the treatment of H5N1 disease.

Key words: H5N1, glycyrrhizin, lung cells, monocyte-derived macrophages

P50

Probing and targeting the NS1/RIG-I interaction of influenza virus A and B

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Influenza viruses are divided into three types (A, B, and C). Influenza A and B are responsible for seasonal epidemics affecting 10–20% of the general population (1); influenza C does not cause epidemics and does not have a severe public-health impact. Many, but not all, of the proteins encoded by these viruses carry out similar function. Here we focus on the function of the viral NS1 protein, which modulates important aspects of the virus replication cycle, including RNA replication, viral protein synthesis and general host-cell physiology (2, 3). The major function of influenza A NS1 is to antagonise IFN-mediated antiviral response interacting with RIG-I, a cytoplasmic sensor of viral RNA. We have used a bacterial reverse two hybrid-system to investigate the intracellular protein interactions that the influenza A virus uses to sabotage the host's pathogen sensors and antiviral effectors. In this system two proteins of interest (X and Y) are expressed as hybrid fusions of a chimeric repressor complex (434 and P22). Dimerisation of X and Y reconstitutes a functional repressor that prevents transcription of reporter genes downstream, resulting in cell death on selective media. Influenza A NS1 was expressed as a fusion protein with 434 while the RIG-I protein (divided in its sub-domain CARD, Hel and RD) and TRIM25 have been expressed as fusion protein with P22. This has enabled the identification of the interaction domain between NS1, RIG-I and TRIM25.
Our preliminary results indicate that influenza A NS1 can interact with both TRIM25 (preventing RIG-I ubiquitinilation) and with RD and CARD domain of RIG-I (preventing the conformational change between inactive and active form). The same method was also used to asses if influenza B NS1 is interacting with RIG-I and we have found that influenza B NS1 also interacts with both RD and CARD domains of RIG-I and with TRIM25.
We are currently using our experience with genetic selection to identify small molecule inhibitors of these key protein-protein interactions (4). The resulting compounds will be used to investigate the role of NS1 in suppressing the intracytoplasmic pathogen sensors of the host (and allowing influenza virus infection and replication to proceed unchecked) and will serve as the starting point for the development of a novel class of anti-influenza therapeutic agents.

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Key words: influenza A and B, NS1 protein, RIG-I

P51

Immuno-genetics of influenza infection in mice

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Abstract: Genetic factors that are associated with increased susceptibility of the host to influenza virus infection or disease are largely unknown. To identify genetic determinants of host susceptibility and resistance, we studied the host response after infection with influenza A virus in various mouse genetic reference populations. We used several inbred mouse strains, recombinant inbred strains (BXD), Interspecific recombinant congenic strains (BcG) and backcross mice, which were infected with influenza A H1N1 (PR8). We further characterized the response in one of the susceptible strain (DBA/2J) and in a resistant mouse strain (C57BL/6J) in terms of viral load, cytokine/chemokine profiles, lung pathology and genome-wide expression analysis. In lung epithelium we found genes, specifically down-regulated in DBA/2J mice. In both mouse strains, chemokines, cytokines and interferon-response genes were up-regulated, indicating that the main innate immune defense pathways were activated. However, many immune response genes were up-regulated in DBA/2J much stronger than in C57BL/6J, and several immune response genes were exclusively regulated in DBA/2J. Compared to C57BL/6J mice, DBA/2J mice exhibited a high viral load, elevated cytokine/chemokine levels and extended lung pathology, which can explain the susceptibility of DBA/2J strain. Infection of recombinant inbred strains (BXD) and F2-backcross mice (B6D2F1 x D2) is currently ongoing to identify QTLs that contribute to the difference in susceptibility to H1N1 virus.

P52

The Influence of targeting an encoded influenza HA-protein to dendritic cells on cellular and humoral immune responses to DNA vaccines

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Introduction. Since Influenza virus infection is a major concern of public health and results in a high morbidity and mortality rate every year worldwide, it is highly desirable to develop vaccines that can be easily adapted to new circulating strains. A promising approach is DNA vaccination followed by electroporation, which provided protection against a lethal influenza challenge in several animal models. Furthermore targeting the encoded antigen to dendritic cells (DC) could enhance the efficacy of DNA vaccination by increased antigen uptake and presentation. The aim of this study was to determine, if the cellular response to conserved epitopes of the influenza hemagglutinin (HA) could be strengthened by coupling the HA to a single-chain antibody directed against the DEC205 receptor and if an induction of specific T-helper cells or cytotoxic T-cells might provide partial cross-protection by limiting the initial spread of the virus and the course of infection.
Methods. Plasmids encoding the single-chain fusion proteins and respective controls were constructed and used for immunogenicity studies in Balb/c mice. Antigen presentation *in vivo* was analyzed by transferring CFSE-labeled TCR-transgenic T-cells into immunized mice to follow the activation of antigen-specific CD4 and CD8 T-cells. Furthermore, naive mice were immunized via DNA electroporation and cellular and humoral immune responses were characterized by intracellular cytokine staining and HA-specific antibody ELISA.
Results. Antigen presentation by MHC class II and consecutive activation of CD4 T-cells were significantly enhanced by the targeted vaccine compared to the controls, whereas both plasmids lead to strong MHC class I presentation. Interestingly, vaccination of naïve mice lead to reduced CD4 and CD8 T-cell responses compared to the non-targeted vaccine. In contrast, the antibody responses were slightly enhanced after two immunizations with the DC-targeted antigens.
Conclusion. Targeting the encoded antigen to DCs alters the response to DNA vaccination with respect to antigen presentation as well as the induction of cellular and humoral immune responses. Despite enhanced presentation, the induction of CTLs and T helper cells seems to be reduced, suggesting the involvement of regulatory T cells.

Key words: DNA immunization, hemagglutinin, single-chain antibody, DC targeting

P53

Human guanylate-binding protein 1 and 3 are induced upon influenza A virus infection and mediate antiviral activity

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Guanylate-binding proteins (GBP) belong to the family of large GTPases and are known to be the most abundant proteins that accumulate in cells in response to IFNγ stimulation. GBP are characterized by high GTPase activity and are therefore involved in various basic cellular processes including signal transduction, cell proliferation and differentiation. Furthermore hGBP-1 has been shown to mediate antiviral activity against vesicular stomatis virus (VSV) and encephalomyocarditis virus (EMCV) in HeLa cells. However, the underlying antiviral mechanism is unknown yet. This prompted us to analyse the role of hGBP-1 and hGBP-3 in influenza virus infected cells. In the course of these studies we identified a splicing variant of hGBP-3 lacking 123 nucleotides at its C-terminus that we termed hGBP-3ΔC (IMV). We observed that expression of endogenous hGBP-1, hGBP-3 and hGBP-3ΔC (IMV) is upregulated in influenza A virus (IAV) infected lung epithelial cells as well as in a variety of epithelial cell lines stimulated with both type I and type II interferons. Furthermore, overexpression of hGBP-1 or hGBP-3 leads to decreased virus titers in A549 cells infected with several human and avian influenza A virus strains, while siRNA mediated knockdown of GBP reverted the antiviral effect. Interestingly, overexpression of hGBP-3ΔC (IMV) seems to decrease virus titers more efficiently than overexpression of hGBP-1 or the full-length hGBP-3. Additionally, overexpression of hGBP-3ΔC (IMV) decreases viral transcription and protein synthesis, assuming that it interferes with an early event of viral replication. We found out that this antiviral effect is due to interference with the viral polymerase complex as the overexpression of hGBP-3ΔC (IMV) leads to a significant decrease in polymerase activity. This was assessed in a recombinant minigenome system with the enzymes of strains A/FPV/Rostock/34 (H7N1) and A/Thailand/1(KAN-1)/2004 (H5N1). Thus, we have identified a novel IFN-induced antiviral acting splice variant of hGBP-3 that acts via inhibition of viral polymerase activity.

Key words: guanylate-binding proteins, influenza A virus

P54

REGULATION OF IFN RESPONSES IN HUMAN DENDRITIC CELLS INDUCED BY PANDEMIC 2009 INFLUENZA A VIRUS

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Since April 2009 a novel swine-origin influenza A (H1N1) virus spread throughout the world. In less than three months after the first cases were reported from Mexico, WHO declared a worldwide H1N1 pandemic. Now one year later the pandemic is interpreted as extinct and the virus continues its world tour as a human adapted seasonal flu. We have studied the activation of innate immune responses and regulation of IFN induction in human monocyte-derived dendritic cells (DC) and macrophages during the infection of 2009 (H1N1) influenza A virus. The pandemic A/Finland/553/2009 virus when compared to the seasonal A/Brisbane/59/07 (H1N1) and A/Beijing/353/89 (H3N2) viruses induced weaker type I (IFN-α/β) and type III (IFN-λ1-3) IFN, CXCL10 and TNF-α gene expression in DCs. Furthermore, different influenza A strains induced the activation of IRF3, IRF7 and NF-κB transcription factors at different extent. Pandemic 2009 and seasonal 2007 H1N1 influenza A strains were inducing weaker IRF3 activation than the H3N2 virus. However, the expression of IRF7 and the activation of NF-κB were clearly slower with the pandemic virus than with the seasonal 2007 virus. The present study demonstrates that regardless of the replication efficiencies of the different influenza A virus strains they have differences in the abilities to induce immune responses. This seems to be due to the variations in the virus recognition induced signaling pathway activation.

P55

Application of Illumina next generation sequencing technology to genotyping of influenza A viruses

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Pigs play an important role in the epidemiology of influenza viruses because they are a "mixing vessel" for the reassortment of gene segments from human, avian and swine influenza virus strains. The emergence of pandemic (H1N1) 2009 virus is further evidence for the importance of pigs as a host. These examples emphasize the necessity for the surveillance of swine influenza viruses.
In the past years, our group characterized nearly 125 influenza A virus strains including avian, swine and human isolates. These viruses were sequenced using the cycle sequencing protocol, a modification of the chain-termination method of Sanger et al. (1977). Sequences were run on a CEQ8000 genetic analysis system of BeckmanCoulter – an eight capillary sequencer.
Since sequencing of 60-80 influenza virus strains per year requires the sequencing capacity of roughly one megabase, we searched for alternative methods. Therefore, a protocol for the fast and reliable sequencing of RNA viruses was established in collaboration with the genome sequencing group of the Fritz Lipmann-Institute, Jena. Using the Illumina/Solexa genome analyzer GAII and the "sequencing by synthesis" technology, this method allows the analysis of 12 virus genomes per lane. Thus, multiplex sequencing of RNA viruses extends the primary applications of this platform which include re-sequencing, gene expression, small RNA discovery and protein-nucleic acid interactions by the phylogenetic analysis of whole genomes of RNA viruses.
After virus sedimentation and RNA preparation, 12 indexed libraries per lane are created. Up to 30 mio reads per lane were obtained in a single run, some 60% of which could be assigned to any of the 12 libraries. Using reference strains as templates for assembly, a varying proportion of reads was mappable to the templates. This proportion ranged from 0.3 to 48% of mappable reads (on average 17%). Mapped reads (6300-690000 per virus) were assembled with ABySS software. Between 17 and 32 contigs per virus were obtained, depending on the quality of the library. These contigs represent 49% to 94% of the genome and corresponds to a 30 – 3300fold coverage per nucleotide. Using an in-house assembly program, 99% of the genomes could be assembled. A blast search of 8697 unmappable contigs identified sequence similarities to GenBank entries of Canis (35,5%), Homo sapiens (10%), Odocoileus (19,9%), Felis, Ovis (2,4%) and Vulpus (3,7%).
Although our protocol is still in development, some 30 viruses have been sequenced with this method including porcine and avian influenza viruses and other RNA viruses.

Key words: RNA sequencing, Illumina/Solexa, swine influenza

P56

Highly pathogenic influenza virus infection of the thymus interferes with T lymphocyte development

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Highly pathogenic avian influenza A viruses (HPAIV) cause severe disease in humans. Still the basis for their increased pathogenesis particularly with regards to the younger population remains unclear. Additionally, the recent pandemic H1N1v outbreak in 2009 demonstrated the urgent need for a better understanding about influenza virus infection. In the present study we demonstrated that HPAIV infection of mice led not only to lung destruction but also to functional damage of the thymus. Moreover, respiratory dendritic cells (RDCs) in the lung functioned as targets for HPAIV infection being able to transport infectious virus from the lung into the thymus. In addition the pandemic H1N1v influenza virus was able to infect RDCs without a proper transport to the thymus. Especially the strong interference of HPAIV with the immune system is devastating for the host and can lead to severe lymphopenia. In summary, from our data we conclude that highly pathogenic influenza viruses are able to reach the thymus via dendritic cells and to interfere with T lymphocyte development. Moreover, this exceptional mechanism might not only be found in influenza virus infection but also might be the reason of the increased immune evasion of some new emerging pathogens.

Key words: H5N1 influenza A virus, pandemic H1N1v, DC, thymus, lymphopenia

P57

CYSTUS052, a new compound against seasonal and pandemic influenza virus

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Influenza still represents a major threat to humans and several animal species. Beside vaccination, only two classes of drugs are available for antiviral treatment against this pathogen. The appearance of pandemic H1N1 and highly pathogenic avian influenza viruses of the H5N1 subtype being able to infect humans reveal the urgent need for new and efficient countermeasures against this disease. Even though several antiviral compounds have been developed against influenza virus, their long-term efficacy is often limited, because of their toxicity or the emergence of drug-resistant virus mutants. Moreover, it is also widely discussed that neuraminidase inhibitors the most common anti-influenza agents, are less effective against new H5N1 isolates and seasonal H1N1 strains. In this regard, we were able to show that a polyphenol rich plant extract from a special variety of Cistus incanus named CYSTUS052 exhibits antiviral activity against influenza viruses *in vitro* and in a mouse model and a randomized, placebo controlled clinical study. The recovery from clinical symptoms was 2.5 days faster in the CYSTUS052 group compared to patients from the placebo group. In addition, we investigated the antiviral potential of CYSTUS052 in comparison to oseltamivir against the swine origin influenza virus (SOIV) H1N1 and various H5N1 influenza viruses. Using an *in vitro* infectivity inhibition assay we found that during the first 24 hours after infection a single treatment of CYSTUS052 was highly effective against these H5N1 viruses compared. Therefore, we conclude that CYSTUS052 might be an effective antiviral with prophylactic and therapeutic potential against influenza viruses including the current pandemic strain and A/H5N1.

P58

The NF-kappaB-inhibitor SC75741 efficiently blocks influenza virus propagation in vitro and in vivo in a mouse infection model

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Influenza remains a formidable foe throughout the world. The appearance of pandemic H1N1 and highly pathogenic avian H5N1 viruses in humans and the emergence of resistant seasonal H1N1 variants against neuraminidase inhibitors highlight the need for new and amply available antiviral drugs. We and others have demonstrated that influenza virus misuses the cellular IKK/NF-kappaB signalling pathway for efficient replication suggesting that this module may be a suitable target for antiviral intervention. Here we show that the novel NF-kappaB inhibitor SC75741 efficiently blocks replication of influenza A and B viruses, including avian and human A/H5N1 isolates in vitro in concentrations that do not affect cell viability or metabolism. In a mouse infection model with highly pathogenic avian influenza viruses A/H5N1 and A/H7N7, we were able to demonstrate reduced clinical symptoms, and survival of SC75741 treated mice. Moreover, influenza virus was reduced in the lung of drug-treated animals. Besides this direct antiviral effect the drug also suppresses H5N1-induced overproduction of cytokines and chemokines in the lung, suggesting that it might prevent hypercytokinemia that is discussed to be associated with pathogenesis after infections with highly pathogenic influenza viruses, such as the A/H5N1 strains. Thus, a SC75741-based drug may serve as a broadly active non-toxic anti-influenza agent.

P59

Infection of well-differentiated porcine airway epithelial cells as target cells for influenza viruses

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Swine play an important role in the epidemiology of influenza viruses. They are considered to serve as "mixing vessels" for the reassortment of genetic material from human, porcine, and avian influenza viruses. Primary host cells of influenza viruses are epithelial cells of the respiratory tract. The continuous cell lines lack several characteristics of the respiratory epithelium so we have established precision-cut lung slices (PCLS) as a culture system for well-differentiated respiratory epithelial cells. PCLS contain the differentiated epithelial cells (ciliated cells and mucus-producing cells) in the original setting. PCLS were prepared with a tissue slicer from the lungs of 3 months old pigs. They are viable as indicated by the ciliary activity observed under the light microscope. In selected samples, the slices were shown to undergo reversible bronchoconstriction by addition of methacholine. The integrity of the cells was also determined by using a Live/Dead viability/cytotoxicity assay kit. PCLS were stained for the presence of sialic acids using lectins from *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA). Anti-nucleoprotein antibody were used for detection of infection by swine influenza viruses (H3N2) and avian influenza viruses (H9N2 and H7N7). The ciliated epithelial cells were found to express α2,6-linked sialic acid prominently, whereas α2,3-linked sialic acid was detected in deeper cell layers but hardly in the ciliated epithelium. Staining of PCLS for viral antigen (NP) indicated that the epithelial cells were sensitive to infection by both porcine and avian influenza viruses. Infection by avian viruses is less efficient as indicated by the amount of infectious virus released into the supernatant. We plan to analyze the adaptation process by performing serial passages of avian influenza virus in porcine PCLS and to determine the mutations assiciated with the adaptation process.

Key words: respiratory epithelium, precision-cut lung slices, influenza viruses, sialic acid

P60

The influenza virus NS1 protein prevents activation of the noncanonical IKK1/p52 NF-κB pathway thereby limiting chemokine expression in lung epithelial cells

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Activation of the canonical IKK2/p50-p65 NF-κB pathway, a major regulator of the innate immune response, was shown to exhibit a virus-supportive rather than an antiviral effect on influenza A virus propagation, a finding that paved the path for novel antiviral approaches targeting this pathway. Besides the canonical NF-κB pathway, an alternative pathway exists that is strongly dependent on NIK and IKK1 kinase activity and regulates processing of the NF-κB2 precursor protein p100 to generate the DNA binding factor p52. Here we investigate whether the noncanonical NF-κB pathway plays a role during influenza A virus infection. Different wildtype virus strains did not induce noncanonical NF-κB signaling in A549 cells. However, a mutant virus lacking the viral NS1 protein readily provoked IKK1-dependent p52 formation, indicating that presence of NS1 suppresses activation of this signaling pathway. Transfection of total RNA from infected cells led to p100 processing suggesting that accumulating viral RNA is the inducing component. To address the question whether activity of the noncanonical NF-κB pathway affects virus replication, siRNA specifically targeting p100 and p52 or a stable cell line overexpressing a dominant negative form of IKK1 (IKK1KD) was used for infection experiments. Determination of virus titers showed no significant effect on viral replication of wt and deltaNS1 virus. Thus the virus-induced noncanonical pathway activation does not seem to regulate cell intrinsic processes relevant for efficient influenza virus replication in infected epithelial host cells. As pulmonary expression of homeostatic chemokines in response to influenza A virus infection has been described earlier in vivo, we next investigated p52 dependent gene expression of CCL19 in response to viral infection. Expression of CCL19 was upregulated upon infection and this was shown to be dependent on functional noncanonical NF-κB signaling. The present results indicate that inhibition of noncanonical NF-κB activation by the viral NS1 protein limits CCL19 expression in virus infected epithelial cells which may be a relevant factor for the systemic immune response.

Key words: noncanonical NF-κB pathway, influenza, NS1

P61

Constitutive Expression Of Chicken Interferon-Alpha in transduced Chickens

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Background: One of the key players in antiviral defence against influenza viruses is the type I interferon (IFN) system. However, the role of IFN during influenza virus infections in birds has to date not been investigated in much detail. In recent studies we could show that IFN treatment before viral challenge failed to protect chickens from highly pathogenic influenza A viruses. Therefore, the question arose whether constitutive expression of chicken interferon-alpha (chIFN-α) would increase virus resistance.
Methods: Using the RCAS (Replication-Competent ASLV long terminal repeat with a Splice acceptor) vector system we expressed chIFN-α1 in chicken embryo fibroblasts (CEFs). Released retroviruses coding for chIFN-α1 or the empty vector were then used to generate transduced chicken embryos. Infection studies were performed *in vitro* and *in ovo*.
Results: CEFs expressing RCAS-chIFN-α1 showed a high degree of resistance to vesicular stomatitis virus and mouse-adapted human influenza A virus strain WSN. In contrast, only a slight delay in the growth of highly pathogenic avian influenza A virus strain FPV Rostock was observed. Substantial levels of IFN activity were detected in heart tissue and allantoic fluid of RCAS-chIFN-α1-transgenic chicken embryos. IFN-expressing transduced embryos survived longer when infected with either WSN or FPV Rostock. Lung homogenates of hatched chIFN-α1 expressing chickens contained enhanced IFN levels, and a serum IFN concentration of about 100 units per ml could be measured during the entire surveillance period of six weeks. RCAS-chIFN-α1-transduced chickens exhibited delayed weight gain and reduced B-cell numbers, but no life-threatening conditions were observed.
Conclusions: Constitutively expressed chIFN-α1 can inhibit the replication of influenza A viruses *in vitro* and *in ovo*, but the protective effect against highly pathogenic virus isolates from chickens is minimal. Retrovirally transduced chicken constitutively expressing chIFN-α1 can hatch but they show some physiological abnormalities. Future work will reveal if survival after viral challenge of chIFN-α1-overexpressing chickens is enhanced.

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P62

Oseltamivir-resistant variants of the 2009 pandemic H1N1 influenza A virus are not attenuated in the guinea pig and ferret transmission models

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Oseltamivir is routinely used worldwide for the treatment of severe influenza A infection, and should drug-resistant pandemic 2009 H1N1 viruses become widespread, this potent defence strategy might fail. Oseltamivir-resistant variants of the pandemic 2009 H1N1 influenza A virus have been detected in a substantial number of patients but, to date, the mutant viruses have not moved into circulation in the general population. It is not known whether the resistance mutations in the viral neuraminidase reduce viral fitness. We addressed this question by studying transmission of oseltamivir-resistant mutants derived from two different isolates of the pandemic H1N1 virus in both the guinea pig and ferret transmission models. *In vitro*, the virus readily acquired a single histidine to tyrosine mutation at position 275 (H275Y) in the viral neuraminidase when serially passaged in cell culture with increasing concentrations of oseltamivir. This mutation conferred a high degree of resistance to oseltamivir but not zanamivir. Unexpectedly, in guinea pigs and ferrets the fitness of the mutant virus was not impaired detectably and both wild-type and mutant viruses were transmitted equally well from animals that were initially inoculated with 1:1 virus mixtures to naïve contacts. Our data suggest that the currently circulating pandemic 2009 H1N1 virus has a high potential to acquire drug-resistance without losing fitness.

Key words: Influenza, oseltamivir-resistance, neuraminidase, viral fitness

P63

Analysis of the sialic acid binding properties of influenza virus hemagglutinins

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The interaction of Influenza viruses with host cells is mediated by the hemagglutinin (HA). This viral surface glycoprotein recognizes sialic acid residues as a receptor determinant. Most avian influenza viruses preferentially bind to α2,3-linked sialic acids whereas human viruses prefer α2,6-linked sialic acids. To distinguish between these two linkages, the lectins MAA and SNA were used in many studies. Due to the huge diversity of oligosaccharides and the different HA subtypes, the two plant lectins are not sufficient to characterize the binding sites of influenza HA.
We have generated soluble HAs that can be used as lectins for detection of those sialoglycoconjugates that are potential interaction partners for influenza viruses. Fusion of the ectodomain to the Fc-component of a human IgG and to a modified GCN4 leucin zipper motif as trimerization domain results in chimeric proteins that are secreted into cell culture supernatant after transfection and were purified by FPLC.
Immunofluorescence binding tests showed a clear binding of the soluble HAs to the cell surface of MDCKII cells which is sensitive to pretreatment with sialidase, showing that the binding is sialic acid-dependent. This effect was quantified by flow cytometry. Binding of the soluble HAs was also detected to the epithelium of the respiratory tract from different species.
Future studies will further evaluate the potential of soluble HAs as a tool to investigate the receptor binding of Influenza A viruses.

Key words: Influenza, soluble hemagglutinin, sialic acids, binding

P64

Oseltamivir and zanamivir susceptibility of swine influenza A viruses isolated between 1981 – 2008 in Germany

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Swine influenza A viruses (FLUAV) represent potential donors of gene segments for human FLUAV. So, the recently emerged pandemic FLUAV of subtype H1N1 comprise the M2 as well as the neuraminidase (NA) gene of European swine viruses. As a result, pandemic FLUAV became amantadine resistant and are neuraminidase susceptible. These recent developments underline the need of antiviral studies with swine FLUAV.
In the present study, NA inhibitor (NAI) susceptibility of ~240 serologically typed swine FLUAV of subtypes H1N1, H1N2, and H3N2 circulating in Germany between 1981 and 2008 was analyzed in chemiluminescence-based neuraminidase inhibition assays. The mean 50% inhibitory concentration of oseltamivir and zanamivir determined for most of these swine FLUAV indicate a good drug susceptibility of tested viruses. They also confirm the subtype specificity in oseltamivir and zanamivir susceptibility, based on differences in 3-dimensional NA structures that may lead to diverse binding affinities of NAI into the enzyme active site. So, H1N1 FLUAV were just as susceptible to oseltamivir as to zanamivir. In contrast, H1N2 and H3N2 viruses were more sensitive to oseltamivir than to zanamivir. Some outliers with 4 or 5 times reduced drug susceptibility were identified. Generally, the results obtained with German swine FLUAV isolates in enzyme inhibition assays strongly correspond with that of human strains.
Plaque and virus yield reduction assays as well as immunohistochemical detection of viral nucleoprotein were applied to compare the inhibitory effect of both drugs against selected isolates of all three circulating subtypes in regard of virus titre and viral spread in MDCK cells. The MDCK cells used in these studies express similar amounts of 2.3- and 2.6-linked sialic acid receptors as do MDCK-SIAT, and NHBE cells (proved by FACS analysis and immunohistochemical staining). Data obtained in cell culture-based assays correlated well with that from enzyme inhibition assays for most viruses. However, some H1N2 isolates that are additionally glycosylated at Asn158 and Asn163 of HA (evidence provided by sequence comparison and detection of glycosylation) as well as H1N1 isolates with changes in receptor-binding sites were resistant to both NAI in MDCK cells. These results suggest that changes in the functional balance between the viral hemagglutinin and the NA may hamper NAI activity against swine FLUAV at least in vitro.

P65

Tuning the efficacy of Influenza A virus nucleoprotein-based T-cell vaccines with cell death-modulating effectors

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Influenza is a vaccine-preventable respiratory disease with high social and economical impact. The current vaccines target the hypervariable membrane proteins hemagglutinin and neuraminidase, necessitating yearly updates of the vaccines. Vaccines based on the internal, more conserved, proteins such as the nucleoprotein (NP) or the matrix protein can induce a heterosubtypic immune response in mice resulting in protection against different strains of influenza viruses. The protective mechanism is often T-cell based, thereby most probably relying on cross-priming and -presentation. It has been shown that induction of cell death can result in more efficient cross-priming events. DNA vaccines are known to be a good means for induction of T-cell responses. By combining NP with the cell death-related molecule RIP-1 or mutants thereof in a single DNA vaccine vector, we investigate the T-cell activation potential of these vaccines, as well as their protective efficacy after lethal challenge with an influenza virus. T-cell activation is investigated by quantification of IFN-gamma-positive T cells (both CD4+ and CD8+) after restimulation. A flow cytometry-based killing assay is used to determine cytotoxic T-cell activity. The absence of morbidity and mortality as well as clearance of virus from the lung after lethal challenge is taken as a read-out for protection and are used to define correlates of protection.

Key words: Influenza, T-cell, cell death, vaccine

P67

Adaptive mutations in the polymerase subunit PB2 of influenza virus A/Hamburg/05/2009 (H1N1) increase polymerase activity and pathogenicity for mice

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Background. Adaption of an avian influenza virus to a mammalian host involves convergent evolution of the polymerase. Over the years, several critical positions for an adaption of the polymerase were identified. In the 2009 pandemic H1N1 virus the critical positions in the PB2 and PA subunits still show the avian signature. It was therefore of interest to find out if adaptive mutations of these positions alter pathogenicity in mammals.

Methods. After setting up a reverse genetic system for the pandemic A/H1N1-virus, we introduced the known adaptive mutations E627K, D701N and S714I and corresponding double mutations in the PB2-subunit of A/Hamburg/05/2009 (H1N1) and analyzed the polymerase activity in mammalian (HEK293T) cells. Recombinant viruses were rescued and tested for pathogenicity in the mouse model.

Results. All adaptive mutations showed an increase in polymerase activity *in vitro.* While the single substitutions did not alter pathogenicity in the mouse model, there was a significant increase after infection with the double mutants.

Conclusion. These observations indicate that adaptive mutations may further increase the pathogenicity of the pandemic H1N1 virus. Thus, its pandemic and pathogenic potential may not be exhausted yet.

P68

Treatment-selected and community-acquired oseltamivir-resistant pandemic A/H1N1 2009 influenza viruses

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Background: In response to the rapid global spread of an antigenically novel A/H1N1 virus which triggered phase 6 according to the global influenza preparedness plan, the World Heath Organization (WHO) recommended surveillance and monitoring for antiviral resistance of influenza viruses.
Materials and Methods: Comprehensive susceptibility analyses of influenza viruses obtained from patients between April 2009 and April 2010 with confirmed pandemic A/H1N1 2009 influenza infections were performed. Therefore, pyrosequencing (PSQ)-based assays for sensitive and high-throughput detection of resistance-associated NA-N1 substitutions H274Y and N294S were designed. Positive controls were generated by site-directed mutagenesis. The sensitivity of these assays was evaluated by testing cDNA samples obtained from viral RNA extracted from respiratory specimens. Altogether, 1570 viruses were analysed for their susceptibility to NA inhibitors oseltamivir and zanamivir, 635 of them phenotypically by fluorometric enzyme activity assay.
Results: The PSQ-based resistance assays distinguish clearly between sensitive wild type and mutant NA. Validation of these assays with classical sequencing, molecular cloning and end-point dilution of plasmid-based positive controls revealed a high specificity and sensitivity allowing analyses on respiratory specimens directly.
Examination of NA susceptibility profiles of 1570 pandemic viruses currently circulating in Germany showed the occurrence of eight (0.5%) oseltamivir-resistant viruses. All these viruses were carrying the H274Y NA substitution, known to confer resistance to oseltamivir. Six of the oseltamivir-resistant infections were treatment-related and detected in patients who were affected by underlying conditions. The selection of resistant viruses during oseltamivir treatment could be documented. The other two oseltamivir-resistant pandemic viruses were isolated from respiratory specimens of immunocompetent and otherwise healthy patients. In the first case, the virus was isolated from a respiratory sample of a 21-year-old man who developed an influenza-like illness while on vacation abroad. The second case was a 9-year-old otherwise healthy boy, who developed influenza like illness and bronchitis after attending a soccer match with approximately 300 other attendees.
All viruses tested remained sensitive to zanamivir.
Conclusion: Oseltamivir resistance was detected in eight pandemic A/H1N1 specimens, 75% of them were associated with oseltamivir administration and co-morbidity. Our findings that severely immunocompromised patients are at a higher risk for selecting oseltamivir-resistant viruses during treatment, confirms previous results and emphasises the importance of monitoring resistance development during the course of treatment. However, our data indicate also the natural emergence or community-acquired oseltamivir-resistant pandemic A/H1N1 influenza infection in immunocompetent and otherwise healthy patients.

P69

Tiam1 mediated Rac1 activation is required for efficient influenza A virus (IAV) replication

Key words: Influenza A virus, Rac1, virion trafficking

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Previously we have shown that the small GTPase Rac1 is activated upon influenza A virus infection. Rac1 is involved in induction of the type I interferon system upon IAV infection by regulation of IRF3 phosphorylation and thereby expression of IFNβ. Interestingly, inhibition of Rac1 activation using a chemical inhibitor as well as knock down by Rac1-specific siRNA leads to reduced rather than enhanced virus titers, thus suggesting additional proviral functions of Rac1. The Rac1 inhibitor NSC23766 specifically inhibits Rac1 GDP/GTP exchange activity by prevention of the interaction of Rac1 with the Rac-specific GEFs Trio and Tiam1. While siRNA provoked knock-down of Trio did not affect virus propagation, knock-down of Tiam1 leads to a significant decrease of progeny virus titers. According to these results, proviral Rac1 activation upon influenza A virus infection involves action of Tiam1. NSC23766 treatment most efficiently blocks virus propagation during the early phase of the replication cycle and seems to result in virion accumulation in the cytoplasm without affecting initial virion internalization, arguing that Rac1 activation via Tiam1 presumably regulates virion trafficking in the endocytic sorting machinery.

P70

Truncated Sequences of Influenza A Virus Subtype H5 Haemagglutinin for Vaccination and Diagnostic Purposes

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Influenza A virus haemagglutinin (HA) is the major target for protective immune responses in the natural host. Vaccines based on conserved antigenic determinants could provide efficient protection from disease and infection and not elicit an immune response against internal viral proteins. On the other hand, serological tests using recombinant immune-dominant proteins devoid of non-specific moieties present in whole cell preparations might have higher sensitivity and specificity. In the present study, four non-overlapping sequences of different functional domains of influenza A virus subtype H5 virus (designated P1, P2, P5 and rHA1) were expressed in yeast *Pichia pastoris* to be used in diagnostic and vaccination purposes. The cDNA of truncated sequences of H5 (A/Thailand/1(Kan-1)/2004) were amplified by PCR using specific primers and cloned into pAOX vector. Linearised plasmids were transformed into *P. pastoris* (strains GS115, SMD1168H) using an optimized protocol. Selected clones were used for secretory expression of polypeptides fused to his-tag. Correct expression was analysed by SDS- PAGE, Western blot and MALDI-TOF. The molecular weight of P1, P2, P5 and rHA1 was 6.5, 7.6, 13 and 39.6 Kda, respectively. P1, P5 and rHA1 polypeptides were purified by nickel affinity chromatography, whereas P2 polypeptide was purified by lectin affinity chromatography. The immunogenicity of these polypeptides was determined in mice and chickens. P1 and rHA1 induced subtype specific antibody titres as analysed by Elisa, immunoflurescent assay (IFA) and microneutralization test (µNT). Moreover, P1 and rHA1 polypeptides were used to develop rElisa for detection antibodies against H5 influenza virus. Twenty five serum samples obtained from vaccinated commercial broiler with commercial inactivated vaccine (H5N2) and twenty-five negative serum samples were analysed with rP1-Elisa, rHA1-Elisa, whole H5N1 Elisa, Western blot, Agar gel immunodiffusion test (AGID) and Haemagglutination inhibition test (HI). rHA1-Elisa proved to be highly sensitive and specific while rP1-Elisa had low sensitivity and specificity compared with Western blot, AGID and HI tests. To study the validity of rHA1 Elisa, further 179 serum samples obtained from broiler chickens previously vaccinated with H5N2 inactivated vaccines, were analysed with rHA1-Elisa, commercial Elisa and HI. The agreement ratio between rElisa and HI was 84.9 % and between commercial Elisa and HI was 76.5 %. In conclusion, *P. Pastoris* may allow development of an effective recombinant influenza vaccine based on truncated sequences of HA that might provide broader protection against H5 viruse. Also, our results demonstrate the potential utility of the rElisa for determination of antibodies to H5 influenza virus.

Key words: yeast expression / avian influenza H5N1/ / recombinant Elisa

P71

Identification and characterization of host factors required in influenza A virus replication

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The influenza A virus (IAV) is one of major pathogens of humans and animals causing annual epidemics or rarely, worldwide pandemics. To complete the viral life cycle, influenza viruses have to use multiple host cell proteins during replication. The identification and characterization of host cellular factors might lead to the development of antiviral drugs against a new pandemic influenza A virus. A lot of cellular factors have recently been identified by a genome wide RNAi-based screen, a useful and efficient method combining systematic gene silencing with an automatic read out system (Karlas, et al., 2010). 287 human host cellular proteins were identified to be involved in influenza A virus replication. Subsequent in-depth investigation of a subset of these factors revealed their life-cycle stage relevance and intriguing mechanistic insights. Importantly, the majority of factors tested were essential for replication of both the highly pathogenic avian H5N1 and the pandemic swine-origin H1N1 influenza viruses, indicating a broad dependency of variant influenza viruses on these factors.
One of the host cell factors that revealed an inhibitory effect on influenza A virus replication upon knockdown was identified as cyclin-dependent kinase inhibitor 1B (CDKN1B). We observed that CDKN1B knockdown cells and CDKN1B-/- mice showed significantly less virus replication. Interestingly, the levels of CDKN1B are phosphorylated upon IAV infections in *in vitro* studies. Silencing of CDKN1B in infected cells reduces the phosphorylation and activation of mitogen-activated protein kinases (ERK1/2), which are essential for IAV replication. We therefore postulate an interaction of CDKN1B with the MAPK pathway. Moreover we will investigate, if IAV induced phosphorylation of CDKN1B leads to cell cycle arrest at G1/S phase and if this is crucial for efficient IAV replication.

Key words: Influenza A virus, genome wide RNAi-based screen, CDKN1B, ERK

P72

New Avian Influenza (H5N1) Outbreak among Wild Birds, Russia, 2010

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Highly pathogenic avian influenza (HPAI) H5N1 virus has been endemic in poultry in South-Eastern Asia since 2003 and widely spread in the world at the moment.

In June 2006, an influenza (H5N1) outbreak was detected in wild birds on the Uvs-Nuur Lake in Western Siberia, Russia. We showed that A/duck/Tuva/01/06, isolated during outbreak, was highly pathogenic for chickens and mice and belonged to the Qinghai-like group (2.2 clade).

In June 2009, an outbreak of HPAI was recorded in wild birds in Mongolia, on the Uvs-Nuur Lake. Phylogenetic analysis of the hemagglutinin gene showed that viruses belong to clade 2.3.2. A/black-headed gull/Tyva/115/09 and A/great crested grebe/Tyva/120/09 viruses are clearly distinguishable from the HPAIVs previously isolated in this Russian region in 2006, A/duck/Tuva/01/06 (clade 2.2), but are close to A/whooper swan/Mongolia/8/2009 and A/whooper swan/Mongolia/2/2009 isolated in Mongolia at the same time.

We hypothesized (EID Journal, Sharshov et al., 2010) that bodies of water like the Qinghai Lake and the Uvs-Nuur Lake may play an important role in the circulation of avian influenza and therefore we continued to study new outbreaks thoroughly.

Our hypothesis was fully confirmed in June 2010. We isolated two HPAI viruses from Great Crested Grebe (*Podiceps cristatus*) and Black-headed Gull (*Larus ridibundus*) on the Uvs-Nuur Lake. A/black-headed gull/Tyva/8/2010 and A/great crested grebe/Tyva/22/2010 viruses were highly pathogenic for chickens and mice and belonged to clade 2.3.2. Phylogenetic analysis of the isolated viruses has shown that these viruses are closely related to those isolated during outbreak in Mongolia in 2009, and isolated from dead birds on Quinghai Lake in 2009.

The intranasal injection of A/great crested grebe/Tyva/22/2010 in mice caused death of the animal. During the experiment we have observed certain neurological signs of a disease in infected animals. Tissue samples from liver, kidney, lungs, heart, intestines and brain were taken for morphological investigation. In all organs we have found extensive disruptions, hemorrhages and blood infusions. Heart muscle has multiple sites of necrosis and disorganization of muscle fiber. In brain multifocal leukocyte infiltration and several sites of blood infusions were found. Nucleus of neurons had dark structures which is common for cell nucleus disrupted with virus.

These viruses, isolated from an outbreak in wild birds have shown pathogenicity and mortality in mice, causing extensive disruption in all organs. Also, neurological signs of disease were described and morphological investigation has shown changes in a brain tissue that have appeared due to viral infection.

Key words: Russia, H5N1 outbreak, wild birds, hotspot, 2010

P73

ADAPTATION OF HEMAGGLUTININ AFTER INTRODUCTION OF A POLYBASIC CLEAVAGE SITE INTO A LOW-PATHOGENIC AVIAN INFLUENZA VIRUS.

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Highly pathogenic avian influenza viruses (HPAIV) differ from all other strains by a polybasic cleavage site in their hemagglutinin (HA). We previously demonstrated that insertion of different polybasic cleavage sites into the HA of the avirulent strain A/Duck/Ukraine/1/1963 (H3N8) (DkUkr63) is not sufficient for immediate transformation into an HPAIV. However, some infected chickens developed temporary clinical symptoms accompanied with cloacal shedding in contrast to the avirulent parent virus. We report here the acquisition of additional adaptive alterations in HA. We re-isolated the DkUkr63 mutant carrying the HA cleavage site from HPAIV A/Chicken/Germany/R28/03 (H7N7) (DkUkr63-R28$_{HACS}$) from cloacal swab and subjected the passaged virus to in-vitro characterization in comparison with its parent. The HA of the passaged DkUkr63-R28$_{HACS}$ carries three amino acid exchanges in the HA: T147S in proximity of receptor binding site, R347K within cleavage site, and I358M corresponding to 10th residue of the highly conserved fusion peptide. Western blot analysis revealed improved HA cleavability. Accordingly, the growth curves on avian (DF1, QT6) and mammalian (A549, Vero) cells demonstrate that the passaged virus has acquired improved replication efficiency. One passage of DkUkr63-R28$_{HACS}$ in chicken led to additional changes in the receptor binding site, cleavage site and fusion peptide, which may contribute to enhanced replication. The influence of each single mutation to it will be elucidated in future studies. Taken together, avirulent avian influenza viruses, in evolutionary stasis in their natural hosts, evolve rapidly under adaptation pressure exerted by an engineered polybasic cleavage site and host change.

Key words: avian influenza, virulence, HA, fusion peptide

P74

Mutation at Position 148 of the NA of a Low Pathogenic H5N1 Avian Influenza Virus after Intracerebral Adaptation and its Importance for Pathogenicity

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Wild birds are the reservoir of all subtypes of low pathogenic avian influenza (LPAI) and transmission of these LPAI viruses is not uncommon. LPAI of the subtypes H5 and H7 can mutate into highly pathogenic avian influenza viruses (HPAI). These HPAI can emerge in poultry and cause high mortality rates in infected flocks.
However, little is known about molecular markers important for virulence and host-range. The main molecular markers identified so far are the HA cleavage site for virulence and the PB2_627 mutation for mammalian hosts. Besides, the importance of glycosylation for both host-range and virulence has been described.
In this study we were looking to identify molecular markers involved in the generation of a HPAI virus from a cloned LPAI H5N1 wild bird isolate (A/Mallard/Italy/3401/2005) after intracerebral (IC)-passaging in day-old SPF-chicken.
Eight IC-adaptation rounds were performed but no highly pathogenic avian influenza virus could be obtained (according to the official definition: IVPI < 1,2). However, a significant increase in the ICPI-value was seen after the first adaptation round. Complete genome sequencing after every adaptation step did not reveal any changes at the level of the HA-cleavage site. However, after the first adaptation round a single mutation was introduced into the NA-148 site, resulting in the loss of a potential glycosylation site. A similar mutation in combination with a carboxy-terminal lysine for the NA-protein has been described to be responsible for an increase the HA-cleavability by interaction with plasminogen for the H1N1 A/WSN/33 virus which is closely related to the H1N1 virus responsible for the 1918 pandemic (Goto and Kawaoka, 1998). The loss of this glycosylation site and its effect on virulence has so far only been described for human influenza viruses. We are further investigating if this NA_148-mutation could also change virulence in an avian isolate by a modified interaction with the plasminogen/plasmin-system.
Attempts to further increase the pathogenicity by IC-passaging were unsuccessful. Possibly the NA_148-mutation and its concurrent increase of virulence decreased the selection pressure and consequently prevented the emergence of an HPAI strain. Moreover, the use of a cloned virus as starting material limited the genomic pool and therefore the adaptive potential. The use of an uncloned viral population for passaging will allow to study the importance of quasispecies populations for mutation from LP to HPAI. Moreover, the ease of mutation at this position will be investigated further.

Key words: Molecular determinants, Pathogenicity, Adaptation, H5N1

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A multiplex assay for the evaluation of avian influenza H5 and H7 neutralizing antibodies directed against haemagglutinin

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The increased number of outbreaks of LPAI and HPAI H5 and H7 influenza viruses in poultry notified worldwide has major public and animal health implications as well as relevant economic impact. In a multiplex format using two forms of luciferase (firefly and renilla), lentiviral pseudotype neutralization assays may be used as a novel diagnostic approach for the simultaneous detection of notifiable avian influenza infections and for the indirect evaluation of vaccine protective efficacy in poultry.
We have constructed H5N1 pp (with A/Viet Nam/1194/04 and A/Indonesia/5/05 HA and NA) using a firefly luciferase reporter and an H7N1 pp (with A/Chicken/Italy/13474/99 HA and NA) using a renilla luciferase reporter.
A panel of sera collected from chickens vaccinated with an inactivated H5N2 vaccine were tested by HI against H5N2, and by H5N1 A/Viet Nam/1194/04 and A/Indonesia/5/05 HA/NA pp assay. Titers obtained via HI correlated strongly with titers obtained by clade 1 A/Viet Nam/1194/04 pp (r^2 = 0.87) and a clade 2 A/Indonesia/5/05 pp (r^2 = 0.83) despite the fact that the HAs were not antigenically matched. A panel of sera collected from turkeys during an outbreak of AI caused by an H7N3 LPAI virus was tested by HI against H7N3 and by A/Chicken/Italy/13474/99 (H7N1) HA/NA pp assay. All sera were positive by HI and A/Chicken/Italy/13474/99 pp assay. A panel of sera collected from chickens vaccinated with an inactivated bivalent vaccine produced with the AI strains H7N1 LPAI/H5N9 LPAI was tested by firefly monoplex assay and by firefly/renilla multiplex assay against A/Viet Nam/1194/04 (H5N1) HA/NA and A/Chicken/Italy/13474/99 (H7N1) HA/NA. Neutralizing antibody titres were found to be equivalent whether measured by monoplex or multiplex assay (which measures H5 and H7 antibody responses within a single serum sample).
We have described a modular multiplex assay that permits the measurement of neutralizing antibody responses against two antigenically distinct HA envelopes in the same serum sample. All serological data obtained with pseudotype assays correlated strongly with HI data. For the sera obtained from chickens vaccinated with a bivalent vaccine the neutralization assay was performed with a single pseudotype virus (H5 or H7) as a monoplex assay or with the two viruses mixed 1:1 (H5 and H7) as a multiplex assay, the neutralizing antibody profiles were found to be equivalent. This multiplex assay is undergoing further evaluation as a routine screening method for the detection of antibodies against notifiable H5 and H7 infections in birds.
Key words: multiplex neutralization assays, lentiviral pseudotypes, avian influenza serology

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STRUCTURE-ACTIVITY RELATIONSHIP AND MECHANISTIC STUDIES OF A NOVEL CLASS OF AGLYCORISTOCETIN DERIVATIVES WITH POTENT AND BROAD ACTIVITY AGAINST INFLUENZA VIRUSES

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We here report on the in vitro anti-influenza virus activity of hydrophobic derivatives of the glycopeptide compound aglycoristocetin. In Madin-Darby canine kidney (MDCK) cells, the lead compound 8e displayed an antivirally effective concentration of 0.4 µM against several strains of influenza A/H1N1, A/H3N2 and B viruses, as determined in a three-day CPE reduction assay using microscopic examination and MTS cell viability assay. The concentration producing 50% inhibition of cell proliferation was 67 µM, yielding an antiviral selectivity index of 167. Virus yield at 72 h was reduced by 3 logs at 5 µM 8e. Analogues derived from aglycovancomycin and aglycoteicoplanin were completely inactive. In addition, the hydrophobic side chain and, in particular, its neutral charge and steric bulkiness, were shown to be important determinants of the anti-influenza virus activity. The narrow structure activity relationship and broad activity against several human influenza viruses suggest a very specific interaction with a highly conserved interaction site. In time-of-addition studies, 8e lost activity when added 1 hour or later post infection, showing that 8e inhibits an early step in virus replication. Inhibition of the hemagglutinin (HA)-mediated membrane fusion is not likely, since 8e produced no effect on virus-induced red blood cell hemolysis at low pH, nor on polykaryon formation of HA-expressing cells. Besides, 8e did not inhibit the conformational change of the HA at low pH, as observed in a tryptic digestion assay. Confocal microscopy on influenza virus-infected MDCK cells stained with anti-nucleoprotein antibody at 1 h after infection, revealed that 8e causes a marked inhibition of the virus uptake into the cells. This inhibition by 8e was more pronounced when the cells were pretreated with the compound during 10 h. Influenza virus fully retained its sensitivity to 8e after eleven sequential virus passages in MDCK cells in the presence of 8e (at concentrations up to 25 µM). The aglycoristocetin derivative 8e represents a new class of potent and broad-acting influenza virus inhibitors with potential therapeutic relevance.
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Key words: influenza, antiviral, glycopeptide antibiotic, ristocetin

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High variability of HA 222 of pandemic influenza A(H1N1) is associated with a fatal outcome

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Background

Since beginning of A(H1N1) pandemic in April 2009 the virus spread worldwide in over 214 countries. The majority of cases caused by this new virus are mild, but also severe including fatal cases have been reported. A D222G substitution was detected in the HA gene with significant frequency in fatal and severe cases. This mutation could cause a shift from α2,6 receptors to the mixed α2,3/α2,6 receptors specificity which increase binding to α2,3 and thus may influence virus tropism and severity of disease. For characterization of pandemic viruses circulating in Germany, we designed pyrosequencing (PSQ) assays and analysed the HA genes in severe and mild cases.

Materials and Methods

RNA was extracted from respiratory specimens or cell cultured viruses. The HA gene was amplified using PCR followed by cycle sequencing and phylogenetic analysis. Based on sequencing data from Germany and worldwide, PSQ assays were developed for identification of HA 203 and 222 variants. Mixed 222 populations were analyzed by T/A cloning and subsequent PSQ analysis.

Results

Phylogenetic analysis of HA genes showed a cocirculation of variant A (S203) and B (S203T) of pandemic A(H1N1) in Germany during 2009/2010. PSQ analysis of about 400 specimens revealed 1% of variant A and 99% of variant B. Further on, PSQ and cycle sequencing analysis of 212 specimens received from mild (164) and severe including fatal cases (48) resulted different HA 222 variants (D/E/G/N222). In mild cases, 6.7% D222E, 0.6% D222N and 0% D222G were detected whereas in severe and fatal cases 6.3% D222E, 0% D222N and 6.7% D222G were identified. Two mixed 222 populations were detected in one severe and one fatal case by PSQ method and confirmed by cycle sequencing and T/A cloning, respectively. PSQ analysis showed that clones from the severe case possess a D/G222 mixture and clones from the fatal case a D/N/G/V/Y222 mixed population.

Conclusion

In this study, D222G substitutions were detected only in severe cases in accordance with studies from Norway and Hong Kong. In addition to known D/G222 mixed populations, a wider variability comprising D, G, N, V, Y is reported here for the first time. Mixed variants might remain the transmission efficiency of the wild type virus but could be able to cause higher infection rates of the lower respiratory tract. The relevance of detected 222 mixed variants (D, G, N, V, Y) with regard to pathogenicity is still unknown and has to be further examined.

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Replication and cytokine induction of pandemic H1N1 and H5N1 influenza A viruses in a human lung explant model

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Human influenza A viruses (IAV) and highly pathogenic avian IAV of the H5N1 subtype propagate efficiently in the human respiratory tract while low pathogenic avian IAV show little replication capability. These differences are unlikely to be explained solely by selective receptor distribution since human airway epithelial cells carry both human and avian receptors. We hypothesize that the host's innate immunity plays an important role in this restriction and that highly pathogenic influenza viruses have evolved mechanisms to evade the human immune system.
To elucidate the contribution of the innate immunity to the species barrier, we established a lung explant infection model from authentic human patient material. In this model, different human and animal IAV were characterized regarding replication and cytokine induction by plaque assay and ELISA, respectively.
Seasonal H3N2 virus as well as H5N1 virus isolated from a fatal human case replicated efficiently whereas a low pathogenic avian and a classical swine IAV of the H1N1 subtype showed only little growth. Pandemic H1N1-2009 virus replicated to a similar extent as a seasonal H1N1 strain and also caused weak chemokine and cytokine responses. In contrast, infection of human lung tissue with a H5N1 virus led to a pronounced induction of various chemokines and cytokines including IP-10, MIP-1beta and IFN-beta, which, remarkably, did not prevent its efficient replication.
Taken together this model provides an experimental approach with high clinical relevance to study IAV infection in the human lung.

Key words: cytokine induction, innate immunity, human lung

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Characterization of HA1 gene of influenzavirus subtype H1N1 circulated from 2000 to 2008 in Liaoning local area

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[Abstract] Objective To characterize HA1 gene of influenza virus subtype H1N1 circulated from 2000 to 2008 in Liaoning local area.
Method Viral RNA was extracted and transcribed into cDNA by reverse transcriptase and amplified by PCR. The products of PCR were purified and sequenced by ABI 3100 Avant. The sequence data were analyzed with epidemic records.
Results （1）The 121 sequences including those from GenBank (except the other earlier 2 sequences) can be roughly divided into two major distinct lineages before and after the year 2000. (2) 4 recent Liaoning strain sequences in 2006~2008 have a variation about 3% compared with the other 101 strains of a few years ago. The nucleic acid and amino acid sequence data of HA1 domain showed that there were four important mutant positions, they were 187 W>R, 188 Y>F，209 Q>K,249 V>A, respectively.
Conclusion The HA1 domain of HA gene of influenza virus (H1N1) isolated from 2000~2008 showed mutation, and the mutated viruses were becoming the dominant circulating strain in Liaoning local area, and showed amino acid sequence difference compared to A/New Caledonia/20/1999, the vaccine components pronounced by WHO from 1999 to 2008, which suggested that further surveillance should be conducted to monitor the virus mutation in circulation.

Key words Influenza virus; subtype H1N1; Sequence analysis

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