Some important aspects of the use of Varicella zoster virus and Influenza virus vaccines

PhD Thesis

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Introduction

Vaccination is the most effective way of preventing infectious diseases. Vaccines currently in use are safe and effective, but neither their safety nor their efficacy is perfect.

Among the factors influencing the impact of vaccines, we investigated the effect of different vaccination routes on the immune response. Among the parenteral vaccination routes the s.c. (subcutaneous) and i.m. (intramuscular) routes are the most commonly used. However, in many studies intradermal (i.d.) route of administration was more efficient and, at lower antigen doses, the vaccine produced an immune response of the same strength as the i.m. or s.c. vaccination at a higher dose. In our study the varicella-zoster vaccine containing a live attenuated virus strain was used (VZV vakcina, Varilrix, GlaxoSmithKline PLC, UK). The varicella-zoster virus is characterized by latency in the sensory dorsal root ganglions of the nerve cells following primary infection. The virus is present in the infected cells, but its replication is partly inhibited, infectious viruses are not produced. Latent virus may reactivate, infectious virus is produced, and zoster disease develops. *In vitro* a low number of infectious particles are released from the infected tissue culture cells because of the attachement of the late endosome mannose-6-phosphate receptors to the viral envelope mannose-6-phosphate in the cells. Most of the viruses are degraded and lose their infectivity in the acidic internal milieu of the late endosome. The ratio of defective:infective particles is 10^4:1 in the supernatant of the infected cells. To prevent herpes zoster, a zoster vaccine containing 14 times more PFU of vOKA (OKA vaccine strain of VZV) than the varicella vaccine, was developed and licensed for the vaccination of immunocompetent subjects older than 60 years in the United States in 2006. Vaccination of immunocompromised individuals with live VZV vaccines can be problematic.

Another factor influencing the effect of vaccines is the antigenicity of the vaccine and its pathogenic role in natural infection. For the protection against viral infections, it may be sufficient to utilize the membrane antigen that binds the virus to its target cells, such as an influenza virus hemagglutinin (HA) protein in the subunit vaccine. However, this finding is true only when the vaccinated individual is infected with a HA-homologous

influenza virus strain. It is not known exactly if the vaccine confers protection, to what degree and by which mechanism in the event of an infection with a HA-heterologous virus strain. In our study with the aluminum phosphate adjuvanted seasonal trivalent inactivated full virion influenza vaccine (Fluval AB) we investigated the potential of this vaccine to protect mice against a heterologous virus strain with humoral components of the sera that are independent of the hemagglutination inhibition (HI), neuraminidase inhibition (NI) and virus neutralizing (VN) activity. In the protection against influenza disease, the HI and NI antibodies against the HA and neuraminidase (NA) proteins have a primary role, but these two proteins contain the most variable parts of the virus. The changes in the HA and NA epitopes cause antibodies to HA and NA produced against the original antigen variant to no longer recognize the virus with the changed HA/NA epitopes, so the antibodies cannot prevent the disease. HA and NA proteins also contain conservative regions, but in the presence of variable proteins their significance is much lower in protection. Likewise, the antibodies produced against the internal proteins of the virus seem less significant in the protection than the HI and NI antibodies. However, antibodies against conservative epitopes cross-react, thus provide cross protections between the different influenza subtypes.

Aims

The aim of our research work was to make suggestions for increasing the efficacy and safety of VZV and influenza vaccines. In our work we wanted to examine the following hypotheses:

1. The efficacy of the immune response can be increased by using an intradermal vaccination route and an inactivated VZV vaccine,
2. Humoral protection against an influenza challenge can be achieved in the absence of HI, NI and VN antibodies
3. After vaccination with Fluval AB, a cellular immune response also develops

Materials and methods

1. The immunological effect of the VZV vaccination after intradermal vaccination

1.1. Experimental animals

Hartley guinea pigs 6 to 8 weeks old were used for the experiments (LAB-ÁLL Bt, Budapest, Hungary). All protocols were approved by the Laboratory Animal Care Committee of the NCE.

1.2. Vaccines and their administration methods

The VZV vaccine (Varilrix, GlaxoSmithKline, Rixensart, Belgium) contains $10^{3.3}$ PFU/0.5 ml of live attenuated VZV (vOKA) propagated in MRC5 human diploid cells. Guinea pigs were immunized i.d. or s.c. with either the 399-PFU or the 1,995-PFU dose of the live or heat inactivated vaccine. A needle-free liquid jet injection- based device that administers liquid into the skin under pressure was developed at the NCE (Division of Virology, National Influenza Reference Laboratory) by Dr. Istvan Jankovics (NCE device) and appropriate needle for the s.c. vaccination.

1.3. To test the accuracy of i.d. administration of the VZV vaccine by the NCE device

Thirteen animals received 0.1 ml of the Varilrix vaccine (399 PFU) by the i.d. route, and 11 animals received the heat-inactivated vaccine similarly. Skin punch biopsy samples from the site of vaccine administration were obtained from two or three animals/time point immediately after vaccination (day 0) and 2, 4, 7, and 14 days later.

1.3.1. Isolation of DNA

Briefly, the skin biopsy specimen was pulverized with liquid nitrogen with a mortar and pestle and 600 ul of RLT-plus buffer was added to this powder. The homogenate was vortexed and then centrifuged. DNA was isolated from the supernatant according to the manufacturer's instructions (Allprep DNA/RNA kit; Qiagen GmbH, Hilden, Germany).

1.3.2. qPCR detection of VZV DNA

The VZV primers used amplified open reading frame 29 of the VZV genome and RNaseP was used as reference gene. LightCycler 480 Probes Master kit (Roche, Mannheim, Germany) was used for the qPCR reaction. The data were analyzed by the LightCycler 480 II system. The comparative Ct method was used for the calculation of the results. The mean value of the samples obtained immediately (time zero) after i.d. administration of the vaccine was used as a reference.

1.4. Measurement of immune responses following the use of various VZV vaccines

Four to six animals per group were immunized i.d. or s.c. with either the 399-PFU or the 1,995-PFU dose of the live or heat-inactivated vaccine. Six guinea pigs vaccinated i.d. with the 399-PFU dose of heat-inactivated vaccine received an i.d. booster with the

same vaccine preparation 23 weeks later. Nonimmunized guinea pigs served as controls. The animals were sacrificed 4 weeks after vaccine administration, and blood and splenocytes were obtained.

1.4.1. VZV antigen preparation and stimulation of splenocytes.

Guinea pig embryonic fibroblast cells from 28-day-old embryos of Hartley guinea pigs, grown in tissue culture flasks, were infected with the live vaccine. On day 5 after infection, cells were scraped than the suspension was sonicated on ice with ultrasonic disintegrator and clarified by centrifugation. Splenocytes were stimulated with the supernatant for 24 h.

1.4.2. RNA isolation and resverse-transcription

Total RNA was extracted from stimulated splenocytes with the RNeasy Plus kit and treated with the RNase-Free DNase set (Qiagen GmbH, Hilden, Germany). The mRNA was reverse transcribed with the Transcriptor First Strand cDNA synthesis kit (Roche, Mannheim, Germany).

1.4.3. Detection of IFN-γ, granzyme B és perforin mRNAexpression by qRT-PCR assay

The LightCycler 480 Probes Master kit (Roche, Mannheim, Germany), the LightCycler 480 II pcr system and the appropriate primers and probes were used to amplify the cDNAs specific for IFN-γ, granzyme B, perforin and as a reference for the RNaseP genes. The comparative Ct method was used for the calculation of the results. The relative expression of the target gene (IFN-γ, granzyme B, perforin) was calculated in relation to a reference sample (the mean of the samples obtained from nonimmunized animals).

1.4.4. ELISA to test the IFN-γ protein level and its correlation with the expression of IFN-γ mRNA

Splenocyte supernatants were assayed in duplicate with an ELISA kit (Guinea Interferon γ ELISA kit; BlueGene Biotech, Shanghai, China). The correlation between the expression of IFN-γ mRNA in stimulated splenocytes and the IFN-γ protein level in the supernatant of the splenocyte cultures was determined by the Pearson test.

1.4.5. VZV-specific antibodies in the sera of immunized animals as determined by VZV gpELISA and virus neutralization assay

Before the ELISA and neutralization assay, the sera from the immunized animals were absorbed with MRC5 cells to eliminate potential anticellular antibodies. VZV

glycoprotein- specific IgG antibodies were determined in duplicate in serial dilutions of the sera of four to six guinea pigs per group with ELISA microplates coated with highly purified VZV glycoproteins (EUROIMMUN anti-VZV glycoprotein ELISA [IgM] kit; EUROIMMUN Ag, Lübeck, Germany), horseradish peroxidase–anti-guinea pig IgG conjugate (DakoCytomation, Glostrup, Denmark), and tetramethylbenzidine-H2O2 substrate from the EUROIMMUN ELISA kit. For the determination of neutralization antibody titers, a plaque reduction neutralization assay was used. Briefly, serially diluted guinea pig sera were mixed with an equal volume of vOKA vaccine in the presence of 10% complement and incubated for 1 h at 37°C. The test was set up such that 45 to 50 PFU was added per well. The antibody-virus mixture was inoculated onto the monolayers of MRC5 cells grown on eight-well tissue culture plates (Asahi Glass Co. Ltd., Tokyo, Japan). The plaques were counted on day 5 with the aid of immunofluorescence with a mouse monoclonal antibody specific for VZV nucleocapsid (Anti-VZV / Varicella Zoster Virus Antibody LS-C76850, Lifespan Biosciences, USA) and an FITC–anti-mouse antibody conjugate (Bartels VRK Anti-Mouse IgG F(ab')2 FITC Conjugate B1029-86B, Trinity Biotech, USA). The antibody titer was calculated as the reciprocal of the dilution that reduced the number of plaques by 50%.

1.5. Statistical analysis

All statistics were calculated with the Microsoft Excel 2007 software package; differences were determined by the Mann- Whitney test. *P* values of _0.05 were considered significant. Correlation coefficients were determined by the Pearson test.

2. The protective efficacy of the influenza vaccination against a HI and NI heterologous virus challenge; cellular and humoral immune responses

2.1. Experimental animals

For the experiments 6-8-week-old female outbred NMRI (National Center for Epidemiology, Budapest) and the inbred Balb/c (Charles River Research Models and Services, Germany GmbH), were used.

2.2. Vaccines and influenza virus strains

Fluval AB (2014/2015) is composed of the H1N1 A/California/07/2009 like NYMC X-179A reassortant strain (min. 15 µg HA), the H3N2 A/Texas/50/2012 like NYMC X-223A reassortant strain (min. 15 µg HA), and the B/Massachusetts/2/2012 wild-type strain (min. 15 µg HA) (Omninvest Ltd., Hungary). The A/California/7/2009 (H1N1)-

like reassortant vaccine strain contains the HA, NA and PB1 genes donated from the A(H1N1) pandemic strain A/California/7/2009 (strain A(H1N1)pdm09), and the other internal genes donated from PR8 virus. The vaccine viruses are inactivated with formalin and adjuvanted with aluminum phosphate. The Fluval AB vaccine is referred to here as TIV+Al. Additional immunogens used for immunization as controls were the TIV not adjuvanted, the TIV+Al combined with complete Freund adjuvant (TIV+Al+F), and the formalin-inactivated mouse-adapted PR8 strain (National Influenza Strain Collection, Hungary), or the inactivated PR8 strain combined with aluminum phosphate adjuvant (PR8+Al), or PBS.

The live A/Puerto Rico/8/34 (H1N1) viruses for the challenge experiments and the live A/California/7/2009 (H1N1)-like reassortant virus for the stimulation of the splenocytes were provided by the National Influenza Center Reference Laboratory (National Epidemiological Center, Budapest), from the influenza strain collection.

2.3. Active immunization and challenge infection

2.3.1. Immunization of NMRI mice; preparing of immune sera for passive immunization; challenge infection

For active immunization six groups of 6-8-week-old female NMRI mice (18 mice/group) were inoculated intramuscularly 3 times at 3-week intervals with the immunogens as follows:

1. TIV at a dose of 45 μg of HA (15 μg of each component)
2. TIV combined with aluminum phosphate adjuvant (TIV+Al, i.e. Fluval AB)
3. TIV+Al vaccine combined with complete Freund adjuvant (TIV+Al+F)
4. Formaline-inactivated PR8 strain at a dose of 15 μg
5. Formaline-inactivated PR8 strain adjuvanted with aluminum-phoshate (PR8+Al)
6. PBS

Blood samples were collected by heart puncture from 5 mice in each group 2 weeks after the last immunization and before the administration of the challenge infection for serum transfer and for determination of HI, NI and VN titers. Thirteen mice/group were challenged by intranasal infection with the live PR8 strain under mild anesthesia. Three mice/challenged groups were sacrificed on Day 6 after the challenge for lung virus titration. The mortality, daily body weight loss and clinical scores were monitored for 10 mice for 18 days after the challenge infection.

2.3.2. Immunization of NMRI mice, cellular immune response

Ten mice were inoculated three times with TIV+Al at weeks 0, 4 and 8. Ten mice were immunized in a similar way, but the third dose of the vaccine was combined with equal volume of complete Freund adjuvant and served as positive controls. At Week 9 the mice of both groups were sacrificed and spleens were obtained for analysis of IFN-γ and granzyme B production in the splenocytes by flow cytometry. Six mice were inoculated three times with PBS and they served as negative controls.

2.3.3. Immunization of Balb/c mice; preparing of immune sera for passive immunization; cellular immune response

In addition to NMRI mice, 6-7 week-old female Balb/c mice in 3 groups of 10 mice were immunized 3 times at 3-week intervals with TIV+Al, or as positive controls, with TIV+Al+F, or as negative controls, with PBS, with vaccine doses described for NMRI mice. Two weeks after the last immunization the mice were sacrificed, and blood samples and spleens were obtained. Serum pools were prepared for adoptive serum transfer and challenge experiments. The splenocytes were used for the determination of IFN-γ and granzyme B mRNA expression by qRT-PCR.

2.4. Passive immunization and challenge infection

From the serum pools collected from actively immunized NMRI or Balb/c mice, 300 µl (NMRI mice) or 100-200 µl (Balb/c mice) diluted with PBS to 500 µl were transferred to each mouse of the 5 to 6 recipient NMRI or Balb/c mice/group via intraperitoneal injection 24 hours prior to challenge with the live PR8 strain. Serum samples from the recipient mice were obtained to compare HI titers in the donor and recipient mice. Then the mortality, daily body weight loss and clinical scores in the passively immunized mice were monitored for 18 days after the challenge.

2.5. Hemagglutination inhibition (HI), Neuraminidase inhibition (NI), Virus neutralization (VN)

Serum antibody titers against the virus strains were measured in HI tests with chicken red blood cells following standard procedures Serum neuraminidase inhibition titers against the virus strains were determined as described. Serum VN assays were carried out on MDCK cells using 100 $TCID_{50}$ of the virus strains/well in a 96 well plate, as described and the virus detection was performed by a HA assay using a chicken erythrocyte suspension.

2.6. Lung virus titers in NMRI mice

Three mice/actively immunized groups were sacrificed on Day 6 after the challenge. Serial dilutions of the supernatants of lung homogenizates were inoculated on MDCK. After a 72-hour incubation period, the number of wells showing cytopathic effects was counted and the $TCID_{50}$ titer per gram of lung tissue was calculated.

2.7. Flow cytometry

Splenocytes were obtained from the NMRI mice on Day 7 after the third inoculation with the TIV+Al or with the TIV+Al+F vaccine or with PBS. Single-cell suspensions were prepared and $2x10^6$ splenocytes were plated onto a 48-well flat-bottomed plate and stimulated for 24 h with the live A/California reassortant strain (MOI=2) or, as control, with culture medium. Brefeldin A (Beckton Dickinson, USA) was added at a final concentration of 1 μg/ml for the last 12 hours of the incubation. For the identification of certain subpopulations, the splenocyte preparations were stained with fluorochrome-conjugated monoclonal antibodies; namely, CD3-FITC, CD4-PerCP, CD8-PE, IFNγ-Alexa Fluor and GrB-eFluor (eBioscience, USA). To count the positive cells, 5000 to 10000 events were acquired by live gating on a FACS Calibur (Beckton Dickinson, USA) and analyzed by means of the CELLQuest pro software package.

2.8. IFN-γ and granzyme B mRNA expression detected by qRT-PCR

Briefly, spleens were obtained from the Balb/c mice on Day 14 after the third inoculation with the TIV+Al or with the TIV+Al+F vaccine or with PBS. Splenocytes were stimulated as described before (2.7.). RNA was isolated from the *in vitro* stimulated splenocytes using the RNeasy Plus Kit (Qiagen, Germany) and transcribed by the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, UK). The LightCycler 480 Probes Master kit (Roche, Mannheim, Germany), the LightCycler 480 II pcr system and the appropriate primers and probes were used to amplify the cDNAs specific for IFN-γ, granzyme B and as a reference for the peptidylprolyl isomerase (PPIA) genes.The relative expression was calculated as the ratio between the mean C_T values of the target gene (IFN-γ and granzyme B) and housekeeping gene in each sample, relative to a reference sample got from PBS-immunized animals.

2.9. Statistical analysis

All statistics were calculated using a Microsoft Excel 2007 software package. Statistical differences between different influenza vaccines relative to the negative control group

were evaluated for survival proportion, change in body weight and clinical score. Body weight data are expressed as the change relative to the Day 0 measurement. Body weight and clinical scores were summarized as a single outcome per animal using an Area Under the Curve (AUC) approach. Mice that died prior to the last day of the observation period were included in the analysis at their last recorded bodyweight and clinical score. Differences were determined by the Mann-Whitney test and p values <0.05 were considered statistically significant. Correlation coefficients were determined by applying the Pearson test.

Results

1. Immunological effect of the VZV vaccine following the i.d. administration of the vaccine

1.1. VZV-DNA in guinea pig skin biopsy specimens at different times after i.d. vaccine administration

To test the accuracy of i.d. administration of the VZV vaccine by the NCE device and to determine the duration of VZV-DNA detectability in the skin, 399-PFU doses of the live and heat-inactivated forms of the vaccine were administered i.d. and skin biopsy specimens were obtained immediately (day 0) and 2, 4, 7, and 14 days later. The contents of VZV-DNA and the housekeeping RNase P DNA in samples obtained immediately after administration of the live (three animals) or heat-inactivated (three animals) vaccine were very similar. In two or three samples per group of animals on the days indicated, the VZV-DNA content declined in time after vaccine administration. On days 4 and 7, the DNA content was higher in the biopsy samples obtained from the animals that received the live vaccine than in those from animals that received the heat-inactivated vaccine ($p>0.05$).

1.2. Expression of IFN-γ, granzyme B, and perforin mRNAs in guinea pig splenocytes after a single administration of a 399-PFU dose of the live or heat-inactivated VZV vaccine and the booster effect of a second 399-PFU dose of the heat-inactivated vaccine given i.d.

The results revealed that the differences in IFN-γ mRNA expression between the animals immunized with either form of the vaccine by either administration route and the nonimmunized animals were not significant. To test the effectiveness of a booster

administration of the 399-PFU dose of heat-inactivated vaccine given i.d., six guinea pigs were given a second dose of the same vaccine i.d. and a significant increase in IFN-γ mRNA expression was observed in the splenocytes compared with that in the nonimmunized animals ($p=0.004$). The granzyme B and perforin mRNAexpression was similar in the animals that received one or two doses of the vaccine and did not differ significantly from that in nonimmunized animals.

1.3. Expression of IFN-γ, granzyme B, and perforin mRNAs in guinea pig splenocytes after a single i.d. or s.c. administration of the 1,995-PFU dose of the live or heat-inactivated VZV vaccine

The expression of IFN-γ mRNA was significantly higher in splenocytes obtained from animals immunized with either the heat-inactivated or the live form i.d. ($p<0.005$) or s.c. ($p<0.05$) than in those from nonimmunized animals. The live vaccine induced a stronger response than the heat-inactivated vaccine by either route of administration, but the difference was not statistically significant. Importantly, the expression of IFN-γ mRNA by splenocytes obtained from animals immunized i.d. with the heat-inactivated form was significantly higher than that by splenocytes from animals immunized with the same vaccine s.c. ($p=0.025$) The expression of granzyme B mRNA in the animals immunized i.d. with the live or heat inactivated vaccine was significantly higher than that in nonimmunized animals ($p<0.005$), while the increase in animals that received these vaccines s.c. was not significant. Importantly, the expression of granzyme B mRNA in animals immunized with the heat inactivated form of the vaccine i.d. was significantly higher than that in animals immunized with the same form of the vaccine s.c. ($p=0.004$). The expression of perforin mRNA was significantly higher only in animals that received the heat-inactivated vaccine i.d. than that in nonimmunized animals ($p<0.005$).

1.4. IFN-γ protein production of guinea pig splenocytes after a single i.d. or s.c. administration of a 1,995-PFU dose of the heat-inactivated or live vaccine s.c.

The content of IFN-γ protein ($p<0.05$) and the fold increase in the expression of IFN-γ mRNA were significantly higher in animals immunized by the i.d. route ($p<0.005$) or by the s.c. route ($p<0.05$) than in nonimmunized animals. Whereas the fold increase in IFN-γ mRNA in animals that received the heat-inactivated vaccine by the i.d. route was significantly higher than that in animals that received this vaccine s.c. ($p=0.025$), the

difference between the IFN-γ protein contents in the supernatants was not significant. Nevertheless, the animals classified as mRNA reactive mirrored the animals classified as IFN-γ protein reactive and the correlation coefficient of the median fold increase in mRNA expression and IFN-γ protein production was 0.88.

1.5. VZV glycoprotein-specific and virus-neutralizing antibody levels after VZV vaccination by the i.d. or s.c. route with the live or heat-inactivated form.

The 1,995-PFU dose of vaccine proved to induce similar VZV glycoprotein-specific IgG antibody titers in guinea pigs immunized with the live or heat-inactivated vaccine either i.d. or s.c., with the nonsignificantly highest level in the animals immunized with the live vaccine s.c. The heat-inactivated vaccine induced a stronger antibody response when administered i.d. than when given s.c., but the difference was not significant. The 399-PFU dose of heat-inactivated vaccine administered i.d. induced a significantly lower titer of glycoprotein-specific IgG antibodies than the 1,995-PFU dose given similarly once ($p<0.05$). The i.d. booster inoculation with the 399-PFU dose of heat-inactivated vaccine produced an antibody response significantly greater than that of the animals immunized with one dose of the same vaccine ($p<0.005$). The neutralization titers showed no intergroup differences in animals immunized with the 1,995- or 399-PFU dose of the vaccines. No neutralization activity was observed in the animals receiving the 399-PFU dose of heat-inactivated vaccine i.d. once, while the animals receiving the same vaccine i.d. two times developed a neutralization titer of 1:32.

2. Protective effect of the influenza vaccination against HA and NA heterologue virus strain

2.1. The impact of immune responses in NMRI mice to TIV+Al on the protection against a challenge infection with a lethal dose of the mouse adapted PR8 strain

All mice injected with PBS died by Day 10 after the intranasal administration of the challenge virus, while all mice immunized with TIV, adjuvanted or not, or immunized with the PR8 strain, adjuvanted or not, survived. The body weight loss after the challenge infection indicated a low-level morbidity of the immunized mice, the mean body weight loss being within 7% in all mice during the observation period with no statistical difference between the immunized groups ($p>0.05$ for all variations) However, there was a strong statistical difference between PBS-inoculated mice and any of the immunogen-immunized groups ($p<0.01$ for all variations). The clinical score was

significantly reduced, and defined as 0 in the groups immunized with TIV, adjuvanted or not, or with PR8, adjuvanted or not, as compared with PBS-inoculated mice, defined as 4 ($p<0.01$). Lungs were removed from three mice in each group on Day 6 after the challenge for titration of the PR8 challenge virus. The results obtained revealed that the virus titers in the lungs of mice immunized with the inactivated PR8 strain adjuvanted or not, were below the threshold of detectability. In mice in the three groups immunized with TIV or TIV+Al or TIV+Al+F, the lung virus titers were similar, with no statistical difference ($p>0.05$) between the three groups. The virus titer in the lungs of mice inoculated with PBS was higher, with a statistical difference of $p<0.05$ between this titer and titers in the lungs of TIV, or TIV+Al, or TIV+Al+F immunized animals.

2.2. The association of protection with serum antibodies in TIV+Al-immunized (Fluval AB) and PR8-challenged mice

To learn whether the observed protection against a lethal PR8 challenge after the immunization of mice with TIV, or TIV+Al, or TIV+Al+F, was associated with humoral response, we carried out passive immunization by transferring 300 µl (NMRI mice) or 100-200 µl (Balb/c mice) of serum pools from groups of actively immunized mice to each naive mouse/groups and then challenged the recipient mice with the PR8 virus strain. In NMRI mice, immune serum obtained from donors immunized with inactivated PR8 virus, or the inactivated PR8+Al combination, or immunized with the TIV+Al+F, conferred protection against death in 100% of the recipient mice ($p<0.01$), while 80% of the recipients survived who received the serum from donors immunized with TIV+Al ($p<0.05$), as compared with the set of mice that received serum from PBS inoculated donors. A non-statistical difference ($p=0.174$) for 20% survival of recipients that received serum from TIV-immunized vs PBS-inoculated mice, but a significant difference ($p=0.047$) for TIV vs TIV+Al-immunized mice was observed. In Balb/c mice 200 µl or 100 µl serum obtained from TIV+Al+F-immunized donors, or 200 µl from the TIV+Al-immunized donors, conferred protection in 100% of the recipients ($p<0.01$), while 100 µl serum from TIV+Al-immunized donors conferred protection only in 50% of recipients ($p=0.021$), as compared with the survival of recipients that received serum from PBS-inoculated mice. A significant difference was seen for the survival of the recipient mice obtaining 200 or 100 µl serum from TIV+Al-immunized mice ($p=0.025$). The body weight loss was significantly less in recipient NMRI mice receiving serum

from PR8- or PR8+Al- or TIV+Al+F-immunized animals (p=0.009) or from TIV+Al-immunized mice (p=0.028), than in mice that received serum from PBS-inoculated donors, indicating that the donor serum contained humoral components that protected the recipient mice not only against death, but also against disease. However, a body-weight loss of recipient mice that received serum from mice immunized with TIV showed no statistical difference as compared with that of the mice who received serum from the PBS-inoculated donors (p=0.117), while it was significantly more severe than that of in mice that received serum from TIV+Al-immunized mice (p=0.047). These results suggest that the combination of TIV with Al-adjuvant was necessary to achieve a sufficiently high titer of humoral components for protection against disease. The body-weight loss in the recipient Balb/c mice was significantly less in animals that received 100 µl or 200 µl serum from donors immunized with TIV+Al+F, or receiving 200µl serum from donors immunized with TIV+Al (p=0.0017), as compared with recipients that received serum from PBS inoculated mice. Body-weight loss was more severe in recipients receiving 100 µl of serum as compared with 200 µl of serum from mice immunized with TIV+Al (p=0.035). During the observation period the clinical score for the recipient NMRI mice that received serum from TIV- or PBS-immunized donors was similarly high (score 4), but it was reduced for mice that received serum from TIV+Al+F-immunized mice (score 1-2), as compared with a score of 0 in mice that received serum from PR8- or PR8+Al -immunized mice. The clinical scores were different (score 1-4) between the individual NMRI mice within the group of recipients that received serum from TIV+Al-immunized donors. The difference in the scores in recipient NMRI mice that received serum from donor mice immunized with PR8, PR8+Al, TIV+Al, or TIV+Al+F, versus scores in recipient mice that received serum from PBS-inoculated mice, was significant (p=0.009). The scores for recipient mice that received serum from TIV-immunized donors showed a significant difference (p=0.028), as compared with that of recipients who obtained serum from TIV+Al-immunized mice. Clinical scores for Balb/c recipients were similar to those for NMRI mice, but for the Balb/c recipients receiving only 100 µl of serum from TIV+Al-immunized donors the clinical scores remained 2 during the observation period, with big interquartile ranges.

2.3. Protection by serum components in mice is not dependent on HI, NI or VN antibodies

To investigate the mechanisms of protection by the immune sera we evaluated the antibody response induced by the TIV against the components of the TIV and against the PR8 challenge virus by HI, NI and VN assays. The serum pools contained high level of HI, NI and VN antibodies against the homologous immunizing strain. However, PR8-specific HI or NI or VN antibodies were not detectable in the sera obtained from mice immunized with the TIV vaccine, adjuvanted or not. Similarly, serum pools from PR8-immunized mice did not cross-react with the A/California reassortant strain. In order to evaluate the success of the serum transfer in NMRI mice, the HI antibody titers against the A/California reassortant strain in the serum samples got from the individual recipient mice before the challenge infection were also determined. HI titers in the recipient NMRI mice were closely correlated with the HI titers in the corresponding donor pool, and were approximately 16-21 fold lower due to serum dilution in the recipient mice. In Balb/c mice, receiving 100 µl or 200 µl of serum/mouse, the HI titer in the recipient mice was 8-32 fold lower than that in the donor pool. High HI levels against the A/California reassortant vaccine strain (GMTs of 126-253 or 160-320 in the serum pool in recipient NMRI or Balb/c mice, respectively) were detected in the recipient mice that exhibited 80-100% protection against death by the PR8 challenge strain and no cross-HI antibody reactivity was observed between the A/California reassortant vaccine strain and PR8 strain in HI, NI and VN assays. Our results told us that the correlation coefficient of the HI titers against the A/California reassortant vaccine strain in the combined groups of the NMRI and Balb/c mice and the survival proportion of the challenged recipient mice was 0.77, and the correlation coefficient for the HI titer and body weight change was 0.71. The positive correlation suggests that the high HI antibody titer specific to the homologous strain is an indication of some degree of protection against an influenza virus infection with no HI cross-reactivity.

2.4. The production of IFN-γ and GrB in *in vitro*-stimulated splenocytes obtained from NMRI mice immunized with TIV+Al (Fluval AB) or, as controls, with TIV+Al+F, as measured by flow cytometry

TIV+Al immunization induced a significant increase ($p<0.05$) in the IFN-γ-producing CD3+/CD4+ and CD3+/CD8+ and CD3-/CD8+ cells and in the granzyme B-producing CD3+/CD4+ cells, as compared with the splenocytes obtained from the PBS-inoculated mice. The presence of IFN-γ or granzyme B-producing cells was higher in all of the

tested splenocyte subpopulations obtained from mice immunized with TIV+Al+F than that in splenocytes obtained from PBS-inoculated mice; with a statistical significance level of $p<0.005$.

2.5. The mRNA expression of IFN-γ and granzyme B in the *in vitro*-stimulated splenocytes obtained from mice immunized with TIV+Al (Fluval AB) or, as controls, with TIV+Al+F, as measured by qRT-PCR

The relative quantification of the IFN-γ and granzyme B mRNA expression in the *in vitro* stimulated splenocytes, obtained from the mice immunized with the TIV+Al or as controls, with TIV+Al+F, demonstrated a 20 to 30-times increase, as compared with splenocytes obtained from PBS-inoculated animals ($p<0.05$ for all combinations). The differences in the extent of mRNA expression in the splenocytes from TIV+Al or TIV+Al+F immunized mice were not significant in any combinations tested ($p>0.05$).

Conclusions

1. I.d. immunization of guinea pigs with the 399-PFU inactivated VZV vaccine induces stronger cellular and humoral immune response, than the same vaccine administered s.c., but the difference is not significant.

   I.d. immunization of guinea pigs with 1995-PFU inactivated VZV vaccine induce significantly stronger cellular immune response, than the same vaccine administered s.c. Humoral immune response is also stronger in the animals immunized i.d., but the difference is not significant.
2. Protection by humoral components of the serum against an influenza virus challenge is achievable in mice in the absence of HI, NI and VN antibodies but the use of the aluminum adjuvant is needed.
3. After vaccination of mice with Fluval AB, a virus specific cellular immune response is also developed.

Publications closely related to the thesis

Sarkadi J, Jankovics M, Fodor K, Kis Z, Takacs, Visontai I, Jankovics I, Gonczol E. (2015). High-level cellular and humoral immune responss in guinea pigs immunized intradermally with a heat-inactivated varicella-zoster virus vaccine. Clin Vaccine Immunol 22:570-577. **IF.: 2.3**

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Other publications related to the thesis

Sarkadi J, Jankovics M, Kis Z, Skare J, Fodor K, Gonczol E, Visontai I, Vajo Z, Jankovics I. (2013). Protection of Chinese painted quails (Coturnix chinensis) against a highly pathogenic H5N1 avian influenza virus strain after vaccination. Arch Virol. 158(12):2577-81. **IF.: 2.282**

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Publications not related to the present thesis

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