Immunologic and Inflammatory Reactions after Safety Trial in Horses with DUVAXYN® IE-T Vaccine

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Summary: Fever occasionally occurs with vaccine safety testing. The purpose of this study was to investigate whether this response is related to components of the inactivated virus or if it is caused by the adjuvant. Ten immune naive foals were randomly allocated to group 1 and group 2. The horses of group 1 (n=5) were intramuscularly injected with an inactivated influenza–tetanus vaccine (Duvaxyn®IE-T). The horses of group 2 (n=5) received a sham vaccine containing the carbomer and aluminiumhydroxyd adjuvant. Both groups were vaccinated second time after 4 weeks of first vaccination. Rectal temperatures were measured daily during the tests (28 days). Blood samples were taken from the horses at 0, 4, 12 hours post first and second vaccination and additionally at 240 hours after the second injection. The protein bands were analysed by SDS-PAGE methods. The fluctuation of the proteins bands was moderate over the time course. The relative mRNA expression of cytokines was measured in peripheral white blood cells (PWBC), using the quantitative TaqMan®Real-Time RT-PCR (qRT-PCR) technique. In conclusion, the adjuvant does not cause fever, but the fever response seen during vaccine testing might be caused by the antigens of equine influenza virus or the tetanus toxoid. Cytokine expression after vaccination was clearly elevated in PWBC. The mRNA-Expression of TNF-α seems to be induced more strongly after the second vaccination in than after the first one.

Key Words: Cytokines, horse, interleukine, qRT-PCR, vaccine.

Atlarda DUVAXYN® IE-T Aşısı ile Yapılan Güvenlik Testinden Sonraki İmmunolojik ve Yangısal Reaksiyonlar

Özet: Aşı güvenlik testlerinde arasıra beklenmeyen bir ateş ortaya çıkmaktadır. Bu çalışmanın amacı, bu ateş cevabının, inaktive edilmiş virus kompenetleriylemi ilişkili ya da bu cevaba adjuvantın neden olup olmadığını araştırmaktır. On adet immune edilmemiş tay rastgele Grup 1 ve Grup 2 ye ayrılmıştır. Grup 1'in atları (n=5) inaktive edilmiş influenza–tetanus aşısı ile (Duvaxyn®IE-T) intramuskuler olarak aşılanmıştır. Grup 2'nin atları ise carbomer ve aluminyumhidroksid adjuvantı içeren 'Yapmacık' (Sham) aşı ile aşılanmıştır. Rektal ısı test süresi boyunca ölçülmüştür. Atlardan İlk ve ikinci aşılama sonrası 0, 4 ve 12 saat ve ayrıca ikinci aşılamadan 240 saat sonra kan örnekleri, atlardan alınmıştır. Protein şeritleri SDS-PAGE metodu ile analize edilmiştir. Test süresi boyunca, protein şeritlerindeki hareketlilik ılımlı seviyede kalmıştır. Periferal beyaz kan hücrelerindeki (PWBC) sitokinlerin mRNA ekspresyonu quantitative TaqMan®Real-Time RT-PCR (qRT-PCR) tekniği ile ölçülmüştür. Sonuçta, ateşin sebebi adjuvant değildir fakat aşı testi boyunca görülen ateş cevabı equine influenza viruslarının antijenleri ya da tetanoz toksoidi tarafından oluşturulmuş olabilir. PWBC deki sitokin ekspresyonu, aşılamadan sonra açıkca yükselmiştir. TNF-α mRNA ekspresyonu ikinci aşılamadan sonra birinci aşılamadan sonrakine göre daha güçlü indukte edilmiş görülmektedir.

Anahtar Kelimeler: Sitokinler, at, interleukin, qRT-PCR, aşı.

Introduction

Equine influenza is a highly contagious disease and is characterized by fever, depression, mucoid or mucoprulent discharge and coughing (27). Influenza outbreaks form an economical thread to the equine industry; hence horses are vaccinated against influenza (3). Vaccination against equine influenza has been practised since more than 30 years, but although vaccines have improved considerably over time, there are continued problems with failure of efficacy under field conditions (22). Vaccination to date has featured intramuscular injection of inactivated virus mixed with adjuvant. This regime is effective for production of systemic antibody and is protective if antibody titres are sufficiently high (3). Whole virus vaccines combined with carbopol adjuvant appeared efficacious so far (18, 20). Before batch release, vaccine producer have to test their products for safety according to European Pharmacopoeia (EP) requirements for inactivated Equine Influenza Virus vaccines. In this test, two doses of vaccine (Fa. Fort Dodge, Weesp, Holland) are administered to each of at least ten foals. After 14 days of the first injection, one dose of vaccine is injected into each of the animals. The animals are observed for a further 14 days. During the 28 days of the test, neither abnormal nor systemic reaction occurs (Monograph 0249, 6). Unexpected and unexplainable hyperthermia, likely fever in its nature, occasionally occurs in this

vaccine safety test with the inactivated equine influenza vaccines. As the tested vaccines likely induce both an inflammatory and an immunologic response at the same time, it is practically impossible to distinguish clinically which of the two reactions actually prevails and to which degree should the reaction be considered as a possible indication of vaccine contamination or other production defects. Since cytokines play a central role in inflammation and immunity, specific cytokines (IL-1, IL-2, IL-4, IL-5, TNF-α -and β) could serve as biomarkers for the involvement of certain processes or cell types (9, 29). Early cytokine transcriptional changes could be useful as a forecasting tool in detection of the fever response. Transcriptional pattern of these cytokines in the early stages of immune response could help to unravel their role in the effect of vaccines.

Several studies indicate that variations in cytokine expression are associated with disease activity in immune-mediated or inflammatory disorders (21). Low concentrations of circulating cytokines in blood or plasma make investigation of systemic cytokine patterns a difficult task. Therefore, sensitive detection systems are required to monitor their expression and secretion under various physiological conditions. Several methods exist that allow quantitation of cytokine expression at the protein level (ELISA, Elispot, biological assays, intracellular cytokine staining (11) and at the mRNA level (Northern blots, in situ hybridization, ribonuclease protection assay, reverse transcriptase polymerase chain reaction (RT-PCR) (5) of peripheral blood mononuclear cells (PBMCs), peripheral blood lymphocyte subpopulations and in tissues (15). The recent introduction of quantitative real-time polymerase chain reaction (PCR) analytic systems facilitates the study of intracellular mRNA transcription by automating amplification and quantification (10, 15). TaqMan® probes rely on the 5`→3` exonuclease of the Taq DNA polymerase which cleaves the fluorescent dye from the probe, thus generating a fluorescent signal with the generation of a PCR product (7, 10). Quantitation can be achieved by monitoring which cycle within the reaction the first detectable fluorescent signal is generated (17). Using this analysis system and the available gene sequences coding for many equine cytokines, it is possible to investigate the effects of many antigens on gene expression in PBMCs.

The purpose of this study was to investigate whether the hyperthermic response occasionally reported after vaccine safety testing is related to the systemic presence of TNF-α, IL-2 and IL-4 and if it is caused by the adjuvant.

Materials and Methods

1. Vaccines

Full vaccine (Duvaxyn IE-T, Fort Dodge Animal Health, Weesp, Netherlands) contained three influenza virus strains; 15 μg HA Influenza A/equi-1 Praque/56 (H3N7), 15 μg HA Influenza A/equi-2 Newmarket-1/93 (H3N8), 15 μg HA Influenza A/ equi-2 Suffolk/89 (H3N8), purified, aluminium hydroxide adsorbed tetanus toxoid and 4 mg Carbomer. *Sham vaccine* (Adjuvant) contained only adjuvant; 0.075 ml 3% Aluminium hydroxide and 4 mg Carbomer 934P.

2. Animals and vaccination

Mean age of 9-month-old ten healthy Haflinger (6) and Noriker (4) horses were purchased from a local ranch and housed in isolation at the research station of the Veterinary University in Vienna. They had a proven history of non-vaccination and were immune naive. The diet consisted of concentrates, hay and/ or grass and/or grass silage. Roughage and drinking water was provided ad libitum. At the start of the trial, foals were allocated randomly to one of two groups. One group (G1) of five animals was vaccinated with an inactivated influenza-tetanus vaccine, whereas the other group (G2) was vaccinated with a sham vaccine and left as a control Group 1 the start of the study (t=0). The foals were vaccinated by deep intramuscular injection in the neck. The foals received two vacci-nations at the start of the study (t=0) and 4 weeks later.

3. Clinical monitoring

Following each injection all horses were observed daily throughout the trial for evidence of adverse local and/or systemic reactions to vaccination or sham vaccination. Attitude, stance and appetite were daily monitored for 14 day after vaccination. In cases clinical abnormalities occurred, they were recorded. During a four hour period after each vaccination horses were monitored for anaphylactic response. Special care was taken to detect possible hypersensitivity reactions such as urticaria, swollen head, severe depression, diarrhoea, ataxia, staggering, rapid respiration, anaphylactic shock and collapse within a few hours after vaccination. Local reactions at the injection site were monitored and scored daily for 14 days after injection. The grade of swelling was recorded daily (0, no reaction palpable or visible; 1, only palpable reaction; 2, swelling less than 5

cm; 3, swelling more than 5 cm). Rectal temperatures were taken daily from day 2 before injection till day 14-post vaccination. Temperatures >38.8°C were regarded as abnormal in these young horses.

4. Sample collection

Peripheral blood samples were collected immediately, 4h and 12h after vaccination. Second blood samples were obtained approximately 4 weeks after the horses had been vaccinated. Blood samples were taken immeditely, 4h, 12h and 240 hours after second vaccination. Bronchoalveolar lavage fluid (BALF) samples were collected 48h before, 24 and 72h after vaccinations.

On each sampling day, blood obtained from jugular venipuncture was collected in EDTA K3 tubes (Vacuette®, Fa. Greiner, Bad Haller, Austria) from each horse between 9:00 am and 21:00 pm. Blood mononuclear cells were isolated by centrifugation (1600 x g 15 min) and washed twice with 1 ml PBS. The serum was separated out by centrifugation at 4 °C, 1600 g for 15 min. The cells were lysed with HL puffer (17 nM TRIS-Puffer, 140 nM NH4Cl, and 50 mM EDTA). Cell pellets were resuspended in PBS and again centrifuged. Following 1 ml Trizol reagent was added in Pellets and stored –20 °C further analysis.

Bronchoalveolar lavage fluids were performed in horses as previously described (28). Horses were sedated with intravenous xylazine (0.5mg/kg) and 2.5m long bronchoalveolar lavage tube was passed through the nasal passages and trachea and wedged into a bronchus 500 ml aliquots of sterile 0.9% saline at 37°C were infused and gently aspirated. Ten millilitres of each BALF sample was submitted to the Central Laboratory, University of Veterinary Medicine Vienna, for total and differential cell counts

For the purpose of establishing the linearity of the Taqman® system (used for real-time RT-PCR) and for the purpose of including a positive control on each Taqman® plate, isolated equine peripheral blood mononuclear cells (PBMCs; 30 million) were from heparinized venous blood by density gradient centrifigation through Ficoll-Hypaque (Leukoprep™, AXIS-SHIELD PoC AS Oslo Norwey). Cells ($3 \cdot 10^5$-$5 \cdot 10^5$ lls/ml) were washed twice and resuspended in lymphocyte growth medium (RPMI-1640 medium) suplemented with 10% fetal horse serum (Gibco Invitrogen, Carlsbad, CA), 100 U/ml penicillin G, 100µg/ml streptomycin (Gibco), L-glutamine (2

mM), 20ng/ml PMA (Phorbol-12-myristate acetate) and 100ng/ml Ionomycin were added to the 10 ml cell suspension and incubated for 24h at 37 °C in a 5% CO_2 atmosphere. Following a 24h incubation period, the cells were isolated by centrifugation at 200 x g for 10 min, re-suspended in PBS and enumerated. Cell pellets were snap frozen and stored at -80 °C.

5. RNA extraction and DNase I treatment

The relative transcription of the mRNA within the cells of the buffy coat was measured using quantitative real-time TaqMan PCR. Total RNA (tRNA) was extracted from lysed cells or PWBCs using Trızol extraction. 1 ml of Trizol was added to pellet. The Trizol-suspension was incubated at 15-25°C for 5 min Add 0,2ml chloroform per 1 ml of Trizol reagent used for the initial homogenization. Incubate samples at 15-25°C for 10 min and centrifuge at no more than 12000 x g for 10 min at 2 to 8°C. The RNA pellet washed once with 75% ethanol. Mix the sample by vortexing and centrifuge at no more then 7500 x g for 5 min at 2 to 8°C. The extracted tRNA was treated with 10 u/µl of Rnase-free DNase I (DNase I, Rnase free 10 units/ml ROCHE) to remove contaminating gDNA at 37°C for 10 min followed by heat inactivation at 95°C for 5 min and chilling on ice. The final volume was adjusted to 120 µl with nuclease-free water and stored at –80°C until use. Production of equine IL-2, IL-4 and TNF-α mRNAs was measured using a qRT-PCR procedure as described previously (4).

6. Primers, TaqMan probes and Quantification of equine cytokine mRNA by real-time TaqMan® PCR

TaqMan®real time PCR was used to study the transcriptional activity of the equine IL-2, IL-4 and TNF-a of PBMCs. Cytokine transcriptional activity was monitored at 0, 4 and 12 h post first and second injection (p.i.) and at 240 hours after the second injection. The design of equine IL-2 and TNF-α primers and probes were previously described (4) and IL-4 primer and probe were previously described (1). The levels of the equine IL-2, IL-4 and TNF-α and housekeeping genes (β-actin, GAPDH and β-2-microglobulin) mRNA (Table 1) were determined using the ABI PRISM™ 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). This detection system uses a fluorogenic 5' nuclease assay (6-FAM), or TaqMan® assay, which allows the real time detection of fluorescence in a polymerase chain reaction (PCR) based assay under universal PCR cycling conditions.

Table 1. Equine primers and probes used for the quantification of cytokines. Labelled with TAMRA™ as the quencher dye and FAM™ as the luminescent dye

Cytokine Amplicon-	Accession No.	Primer	Sequence (5`→3`) Lenght (bp)
IL-2 GAT	X69393 124	Forward	CAA GAA TCC CAA ACT CTC CAA
		Reverse	TCG AGA GAA AGT TTT TTA GCA TTT CC
		Probe	CAT GCC CAA GAA GGC CAC AGA AT T G
IL-4	AF305617 74	Forwad	TCG TGC ATG GAG CTG ACT GTA
		Reverse	GCC CTG CAG AT TCC TTT CC
		Probe	CCT TTG CTG GCC CGA AGA ACA CAG A
IL-α	M64087 101	Forward	GCC CAG ACA CTC GAT CAT CTT C
		Reverse	CAT TTG CAC GCC CAC TCA
		Probe	CCC ATG TTG TAG CAA ACC CCC AAG C
β-actin	AF035774 68	Forwad	AGG GAA ATC GTG CGT GAC A
		Reverse	GCC ATC TCC TGC TCG AAG TC
		Probe	CAA GGA GAA GCT CTG CTA TGT CGC CCT
β-2-micro- globulin	X69083 92	Forwad	TCGTCCTGCTCGGGCTACT
		Reverse	ATTCTCTGCTGGGTGACGTAG
		Probe	CGT CCC GCG TGT TCC GAA GGT T
GAPDH	AF097178 70	Forwad	TCG CCA TCA ATG ACC CCT T
		Reverse	CCA TGG GTG GAA TCA TAC TGA A
		Probe	CCT CAA CTA CAT GGT CTA C

Exon-exon junctions are underlined

Except for the equine GAPDH TaqMan [®] MGB Probe that was kindly provided by Applied Biosystems (Forster City, CA), all primers and probes used in this study (sequences listed in Table 1) were synthesized by MWG Biotechnology and designed using the Primer Express® sofware (Applied Biosystems, Foster City, CA). The endogenous "housekeeping" genes (β-actin, GAPDH and β-2-microglobulin) were also quantified, and results were normalized to these values. A housekeeping gene, which is present at constant amounts in all samples, can be used to correct for these minor variations (23).

This was achieved by constructing, for each PCR run, a standard curve from serial dilutions of a purified DNA. The internal probe was labelled at the 5`end with the reporter dye FAM (6 - carboxyfluorescein), at the 3` end with the quencher dye TAMRA (6-carboxytetramethyl-rhadomine) and was phosphate blocked at the 3` end to prevent extension. Briefly each equine cDNA was assayed for the cytokine profile and for house keeping gene (β-actin, GAPDH and β-2-microglobulin) as an endogenous control in separate wells in 25 µl PCR mixtures containing final concentrations of 400 nm primer, 80 nm probe and commercially available PCR mastermix (TaqMan® Universal PCR Mastermix, PE Biosystems, Foster City, CA) containing 10 mm Tris-HCl (pH 8.3), 50 mm KCl, 5 mm MgCl$_2$, 2.5 mm deoxynucleotide triphosphates, 0.625 U of AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 10 µl of the diluted cDNA sample. The samples were placed in 96 well plates and amplified in an automated fluorometer (ABI Prism 7700 Sequence Detection System, PE-Biosystems). Amplification conditions were 30 min at 42°C, 10 min at 95°C, 45 cycles of 15 s at 95°C and 60 s at 60°C. The cytokine transcripts were calculated according to the comparative threshold cycle method (15).

7. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by using the method of Laemmli (14). Briefly, serum diluted 1:10 was electrophoresed at 13°C on a 10-15% SDS-PAGE gradient gel. Samples were loaded after reduction with dithiothreitol. SDS gels were run for 46-60 min with 200 V at room temperature. After electrophoresis, gels were stained with Coomassie Blue R-250. Molecular weight prestained standards (LMW-Marker) were used to estimate molecular size of the protein bands identified.

8. Statistical analysis

White blood cell counts were analysed by Mann-Whitney U-test. Rectal temperatures were assumed to be normally distributed and analysed by ANOVA. Because in some horses expression was very weak, the cytokine activities were not statistically analysed, since the number of cases

was to low to make meaningfull statistical inferences. The level of significance for tests was set at $p < 0.05$. SPSS 11.5 statistical software program for Windows was used for the calculations and tests.

Results

1. Pyrogenicity in Horses

Mean group temperatures are displayed in Figure 1a and 1b. All G1 horses had a clinical relevant increase of rectal temperatures >38.8 °C at 12 h post first injection. Thereafter their temperatures declined; however, the mean body temperature remained over base line for a further 48 h and then dropped to baseline levels at 72 h. None of the G2 horses had clinical relevant increase of rectal temperatures at any moment after the first injection, except one horse (nr 3) that had a sudden rise in temperature to 40.3 °C at 3 days post injection.

After the second injection, temperature response in G1 was already clinically relevant after 4 h and at 12 h. Maximal temperatures in G1 ranged from 39.1 to 40.1°C after first and from 40.8 to 41.0°C after second injection.

Horses in G2 did not show a pyrogenic response at any moment after second injection. After the first injection, mean temperature in G1 was significantly higher at 12 h (p=0.001) and 24 h (p=0.005) p.i. than in G2. After the second injection, mean temperature in G1 was significantly higher at 12 h (p=0.005), 24 h
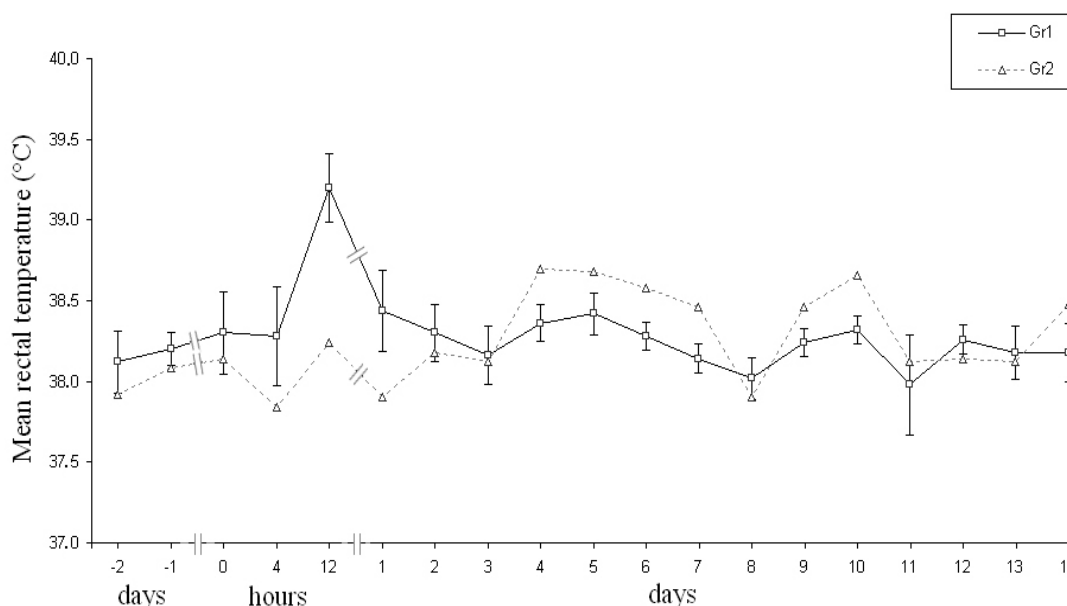
Figure 1.a. Daily mean rectal temperature after first vaccinations. (G1= vaccinated animals; G2 = adjuvanted animals). All animals of Group 1 were vaccinated at 0 hours with a full vaccine against equine influenza. All animals of Group 2 were vaccinated at 0 hours with a sham vaccine against equine vaccine
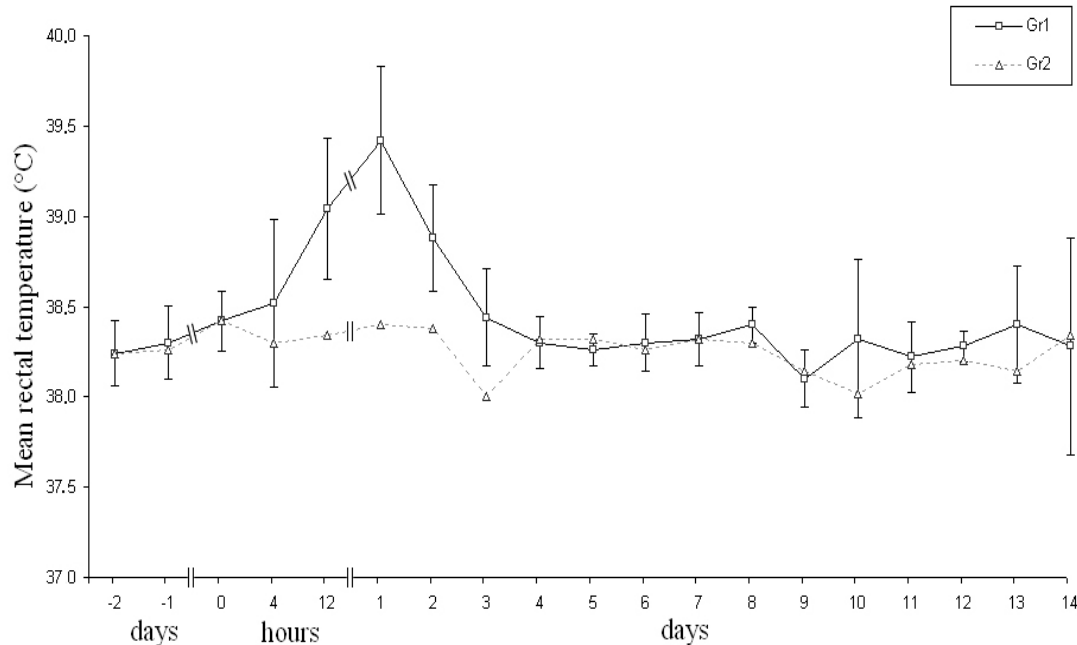
Figure 1.b. Daily mean rectal temperature after second vaccinations. (G1= vaccinated animals; G2 = adjuvanted animals).

(p=0.001), 48 h (p=0.011) and 72 h (p=0.039) p.i than G2.

2. Clinical symptoms

No systemic reactions were observed in the vaccinates or in the controls for fourteen days post vaccination, except one horse of the G1. Only in 2 horses low grade rapidly resolving swellings at the injection site were noticed in G2 horses. The white blood cell counts are shown in Table 2. Total leukocytes counts were significantly increased at 12 hours after second injection in G1 (p=0.018) compared to other sample moments. Granulocyt counts were also significantly increased (p= 0.001) 12 hours after second injection in G1.

3. BALF

In the statistical evaluation of the balf cells could not be found any significant difference between the two groups. The percentage parts of the balf cells of the two groups are shown in Table 3.

4. Cytokine mRNA expression

The patterns of mean cytokine mRNA expression are shown in Figures 2 trough 4.The mRNA of the following cytokines was expressed in the PBMCs: IL-2, IL-4, and TNF- α. The expression of mRNA for IL-2, IL-4 and TNF- α were similar in horses from both groups at 0 h (baseline). The IL-2 expression pattern after the first injection in both groups was the same. At 4 hours IL-2 expression was highly increased. After the second vaccination the patterns were not equal, mean IL-2 expression in G1 horses was mildly increased at 4 and 12 hours post injection, while mean activity in G2 horses was roughly 10-fold lower than G1 horses at 4, 12 and 240 hr post injection (Fig 2).

The IL-4 mRNA expression pattern in both groups after the first injection was similar, but mean IL-4 expression in G1 horses was mildly increased at 12 hours after the second vaccination. G2 horses showed moderately elevated mRNA expression already at 4 h and 12 h post injection. At the moment of 240 hours after the second injection was the expression at horses from group 1 clearly increased while it had been decresed the initial level at animals of the group 2 (Fig 3).

TNF- α expression after first injection in both groups was mildly increased at 4 h and 12 h after the first injection. It dropped to baseline levels in G1 horses and remained mildly elevated in G2 horses at 12 h post injection. After the second injection, TNF- α expression was slightly increased at 4 and 12h post vaccination in Group 1. After the second injection became the both groups

Table 2. The Mean ±S.D number of nucleated cells and proportion of cell types in blood. G1 is full vaccine group, G2 is sham vaccine group

Sample time	Total nucleated cells (µl)	Neutrophils (µl)	Lymphocytes (µl)	% Monocytes	% Eosinophiles
G1 0h pre vacc.	14240 ± 1542	6314 ± 810	6519 ± 1290	5.7 ± 1.0	2.12 ± 0.5
G2 0h pre vacc.	11968 ± 1027	5567 ± 1663	5194 ± 1575	6.2 ± 0.7	1.5 ± 1.0
G1 12h post vacc.	16976 ± 3213*	11291 ± 2316*	4538 ± 1190	4.6 ± 0.8	1.0 ± 0.3
G2 12h post vacc.	12444 ± 1126	5764 ± 1096	5597 ± 1937	5.4 ± 1.0	1.5 ± 1.1
G1 48h post vacc.	14348 ± 2377	6555 ± 2204	6066 ± 1386	7.7 ± 0.9	2.0 ± 0.2
G2 48h post vacc.	11455 ± 848	5631 ± 975	4841 ± 1338	5.1 ± 1.1	1.6 ± 1.3

* Indicates p0.05 relative to controls at that sampling time

Table 3. The Mean number of nucleated cells and proportion of cell types in BALF. G1 is full vaccine group, G2 is sham vaccine group

Sample time	% Neutrophils	% Monocytes	% Eosinophiles
G1 0h pre vacc.	5.70	94.00	0.30
G2 0h pre vacc.	0.50	99.50	0.00
G1 12h post vacc.	11.90	87.90	0.20
G2 12h post vacc.	12.00	87.70	0.30
G1 48h post vacc.	12.00	86.40	1.60
G2 48h post vacc.	11.80	85.30	2.90

increased TNFα mRNA-expression in comparison to the 0 hour value, at which the expression is stronger at Group 1 than at Group 2. TNFα mRNA-expression in both groups seems to have returned to the basal value at the moment of 240 hours after the second injections (Fig 4).

5. SDS-PAGE

The protein bands of the Group 1 were similar to the protein bands of Group 2. The molecular weights of these five bands between 14 and 94 kDa were estimated at 20, 30, 43 and 67 kDa (Fig. 5).

Discussion and Conclusion

This study was carried out to investigate the normal post vaccinal reactions after vaccination with inactivated equine influenza vaccine.

Sham vaccinated horses did not show any clinical significant elevation of body temperature. In contrary, all full vaccinated horses indicated a pyrogenic response after each injection. Since carbopol and aluminium hydroxide was present in both test preparations, the pyrogenic response of the whole vaccinated horses might have been caused by molecules of the influenza virus and /or tetanus toxoid. The local interaction of the whole
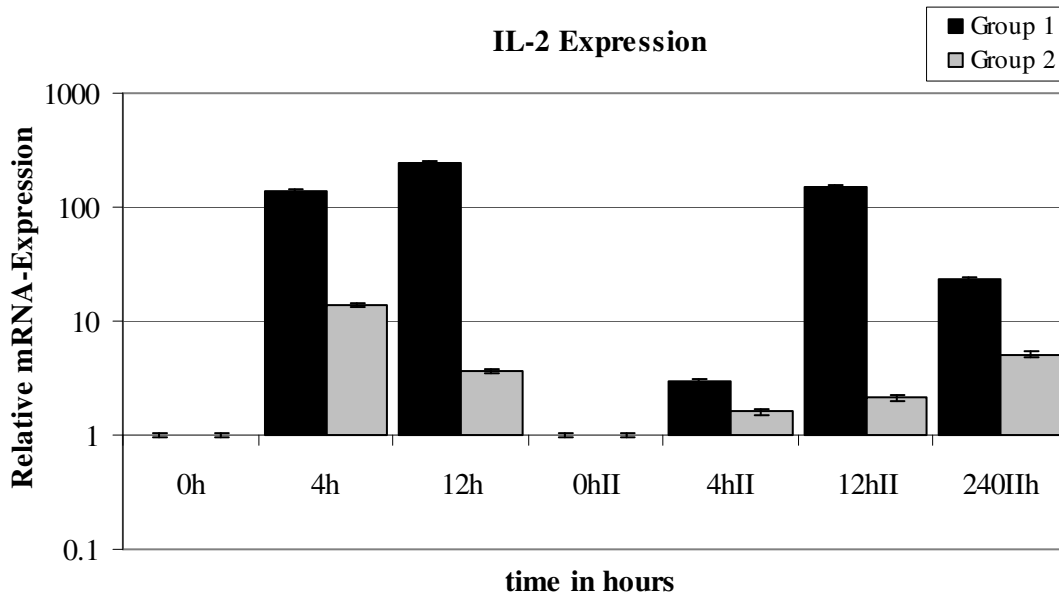
Figure 2. IL-2 mRNA Expression in blood cells of G1 (full vaccinated) horses and G2 (sham vaccinated horses)
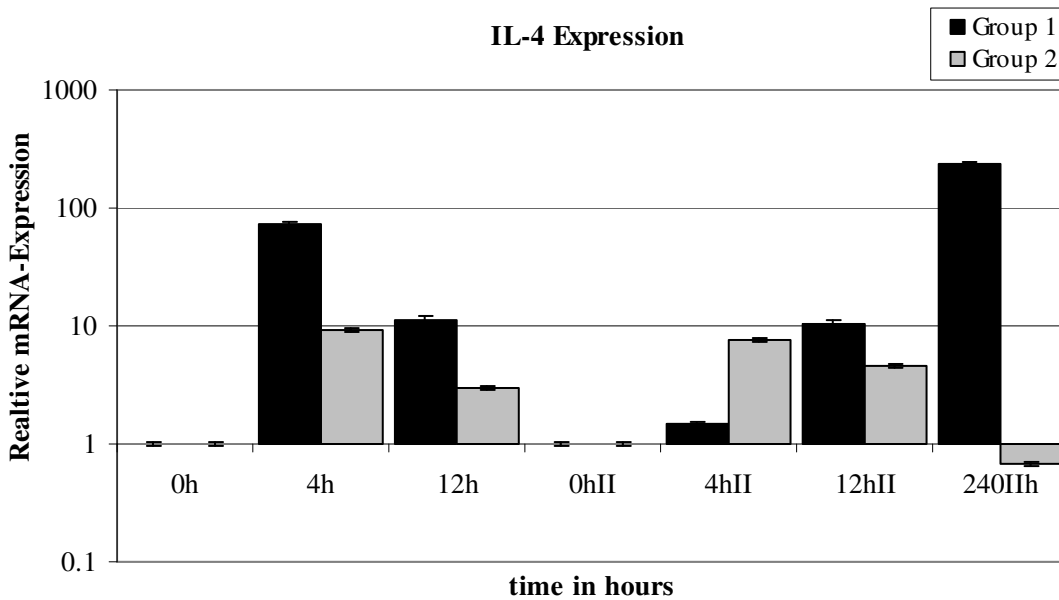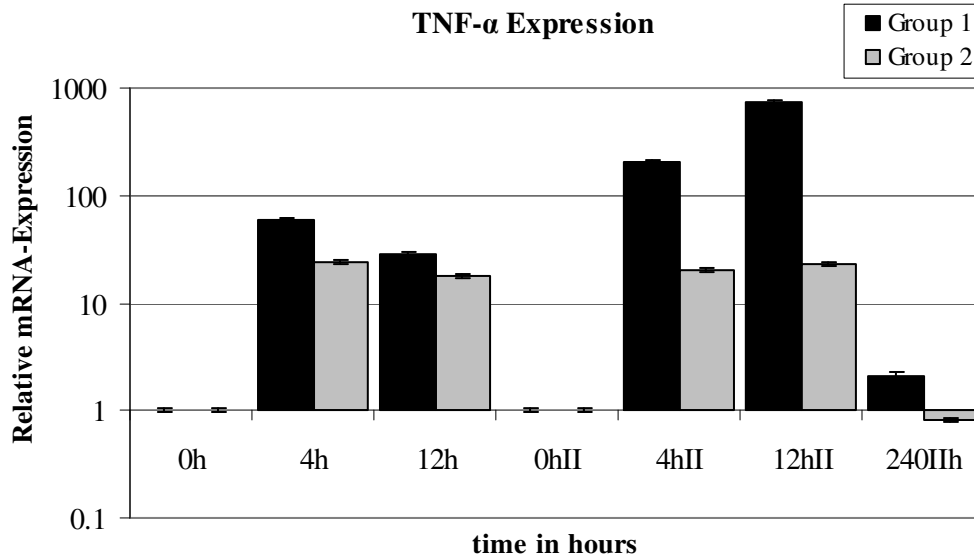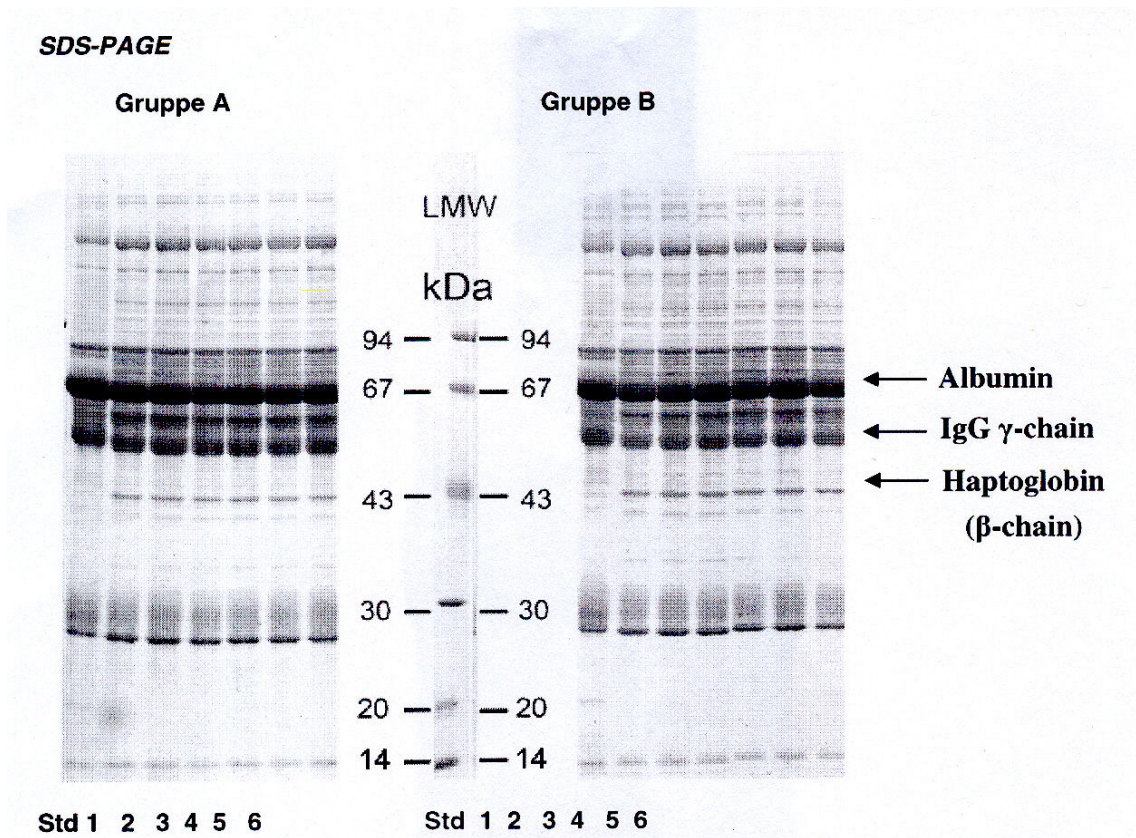

Figure 3. IL-4 mRNA Expression in blood cells of G1 (full vaccinated) horses and G2 (sham vaccinated horses)

TNF-α Expression

Figure 4. IL-2 mRNA Expression in blood cells of G1 (full vaccinated) horses and G2 (sham vaccinated horses)

Figure 5. Lane 1: 0h after I.Vaccination, Lane 2: 4h after I.Vaccination, Lane 3: 12h after I.Vaccination, Lane 4: 0h after II. Vaccination, Lane 5: 4h after II. Vaccination, Lane 6: 12h after II. Vaccination

vaccine components with APC might induce cytokine production and release some cytokines which lead to a hyperthermic response in the hypothalamus by directly or via stimulation of other cells.

The statistical analysis regarding the BALF cells revealed no significant differences in the percentage of distribution for the examined cells. The application of whole- and shamvaccines did not cause any alterations on the leucocyte counts in the BALF. Therefore, no significant effect of the virus antigen was determined. Similarly, any significant noticeable effect on the course of the acute phase proteine was observed. The vaccination with whole vaccine caused the granulocytosis, while the shamvaccine did not.

Since cytokines are the messengers of the inflammation and immune response, we preferred to study cytokine patterns in blood cells of immune stimulated horses. However cytokine research in veterinary field is severely restricted due to the lack of species-specific monoclonal antibodies (24). Some progress has been made by adapting bioassays for a few cytokines such as IL-1, IL-6 and TNF-α that function in a non-species-specific manner (16,19,25), but these bioassays are cumbersome and have a low specificity. Moreover some domestic animal cytokines do not exert activity in murine cells used in the tests. Species-specific monoclonal antibodies (MAbs) and cytokine standards are not yet available for sufficient cytokines to permit comprehensive studies using immunoassays (8). Furthermore, although some equine cytokines can be quantified at protein level, it is diffucult to measure the changes of these cytokines in the systemic blood, because they have low protein concentrations, and partially extreme short half life. A way around this problem the cytokine mRNA could be analysed in the blood cells. Kruse *et al.* (13) and Stordeur *et al.* (26) described the suitability of a RT-PCR based cytokine measuring technique to monitor immune response in white blood cells.

For the endogenous control of the qRT-PCR the housekeeping genes β-Aktin, GAPDH and β-2-microglobulins were used. The normalizing with a standard is necessary to avoid mistakes in the qRT-PCR which can be achieved due to different start material quantities in the different RNA samples. This applies particularly in the case of samples of different individuals (2). During the relative quantification the selection of a suitable internal control is an important aspect. The reference gene should be constantly expressed and not be influenced through the test conditions (2,12). These housekeeping genes are used frequently for the expression analysis as standard control from target genes, as for example IL-2, IL-4 and TNF-α. All Ct-values of housekeeping-genes were evaluated and they were not appropriate for internal standard, because the Ct-values were strongly variable. For this reason the expressions of the target genes were normalized to cell amounts. The use of the housekeeping genes is controversial as internal standard, because β-Aktin and GAPDH exist processed pseudo genes (intronfree copies) (2,30).

It is shown, that the vaccine Duvaxyn®IE-T induces the cytokine mRNA expression in equinen PBMCs. The intramuscularly application of the vaccine led to an increase of the cytokine mRNA expression in the PBMCs of the examined horses. After intramuscular injection of the full vaccine and the during the subsequent pyrogenic response (Fig. 1a and b), some indication of increased IL-2 and IL-4 activity was seen, especially after second vaccination. We anticipated to see a clear elevated expression of TNF-α mRNA as cause for the pyrogenic response, however the increased expression after first injection was not very spectactular. On the other hand, IL-4 mRNA expression, that is accosiated with Th2-type involvement, appeared to be induced by the antigen components after the vaccinations. Its appearance may be associated with memory cell activation and trafficing of homing lymphocytes. The slightly elevated expression of IL-2 at 4 and 12 hours in G2 horses receieved their second carbapol injection was probably merely random variation.

In conclusion, the fever response of the vaccinated animals with DUVAXYN IE T can be explained by a stimulation of the thermocenter in the hypothalamus associated with the releasing of TNF-α. However, other factors could be responsible for the short-term temperature rise. The fever response in the patients could be caused by the contamination of vaccines such as bacterias, virus or fungus, beside stress situations might be effective.

In order to be meaningful as safety guideline, this study indicates that Monograph 0249 of the European Pharmacopoeia should be reworded such that it includes a statement what could be considered as acceptable normal biological post vaccinal reaction.

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