Clinic for Poultry
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Studies on the pathogenesis of avian Metapneumovirus (aMPV) infection in turkeys and on the protective effect of a microparticle-based, genetic engineered aMPV vaccination strategy

THESIS

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Meiner Familie

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Publications

Research articles:

LIMAN, M. & S. RAUTENSCHLEIN (2007):
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LIMAN, M., L. PEISER, G. ZIMMER, M. PROPSTING, H. Y. NAIM & S. RAUTENSCHLEIN (2007):
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Avian Pathol. 37, 65-74

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8th Avian Immunology Research Group Meeting (Munich, Germany; 4th – 7th September 2004)

List of abbreviations

aa	amino acid
aMPV	avian Metapneumovirus
APC	antigen presenting cells
ART	avian rhinotracheitis
BSA	bovine serum albumen
CD_{50}	ciliostatic dose
CD 4 or 8 (+)	cluster of differentiation 4 or 8 (positive)
CEF	chicken embryo fibroblasts
CEL	chicken embryo liver cells
CMI	cell mediated immunity
CpG	cytosine-phosphatidyle-guanosine
Con A	concanavalin A
CRS	controlled release system
CT	cycle threshold
CTAB	cetyltrimethylammonium-bromide
CTL	cytotoxic T lymphocyte
DCM	dichloromethane
ELISA	enzyme-linked immunosorbent assay
F	fusion (protein)
FBS	fetal bovine serum
G	(attachment) glycoprotein
GFP	glow fluorescence protein
GMT	geometric mean titre
hMPV	human Metapneumovirus
HG	Harderian gland
IFN (γ or I)	interferon (gamma or type I)
Ig	immunoglobulin
IL	interleukine

iNOS	inducible NO-synthetase
kb	kilobase(s)
kDa	kilo-Dalton(s)
L	large RNA-dependent RNA polymerase
LPS	lipopolysaccharide
M/ M_2	matrix/ second matrix (protein)
MHC	major histocompatibility complex
MP	microparticles
MP-BSA	microparticles encapsulating BSA
MPpF	pF-loaded microparticles
MPpM	empty plasmid vector-loaded MP
MPrecF	microparticles encapsulating recF
mRNA	messenger ribonucleic acid
N	nucleocapsid (protein)
NK	natural killer (cells)
NO	nitric oxide
NOIF	NO inducing factors
NS_1 & NS_2	non-structural (protein) 1 and 2
P	phosphoprotein
PBS	phosphate-buffered saline
PC	post challenge
PEI	polyethyleneimine
pF	plasmid DNA encoding for aMPV F protein
PI	post inoculation/ infection
PLGA	poly ($_{D,L}$-lactic-co-glycolic acid)
PVDF	polyvinylidene fluoride
recF	recombinant aMPV F protein
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase polymerase chain reaction
QRT-PCR	quantitative real time RT-PCR

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH	small hydrophobic (protein)
SHS	swollen head syndrome
(S/P)-ratio	sample to positive ratio
SPF	specific pathogen free
$TCID_{50}$	tissue culture infectious dose
Th	T helper (cells)
TOC	tracheal organ cultures
URT	upper respiratory tract
VN	virus neutralization
VNT	virus neutralization testing
VSV	vesicular stomatitis virus

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1. Introduction

Infections of the respiratory tract and enteric disorders have significant economical impact on poultry production worldwide. Respiratory diseases may result in dramatic losses in parental stocks, layers and grow-out poultry. In the last decades the industrialization of poultry production combined with veterinary prophylactic measures and hygiene has continuously increased. Due to that the control of the classical monocausal diseases has been more successful. With a decrease in monocausal infections new emerging syndromes and factorial diseases of complex or unknown etiology have become a thread to the poultry's health status.

Turkey Rhinotracheitis (TRT) was first observed in 1978 (BUYS u. DU PREEZ 1980) and was thought to be a syndrome of multicausal etiology for many years. Although the causative agent was later identified as the avian Metapneumovirus (aMPV) (MCDOUGALL u. COOK 1986), TRT still should be considered as a syndrome. The impact of aMPV-infection on turkey's health is significantly influenced by a variety of additional factors, such as secondary bacterial infections and environmental stress. The causative agent aMPV may induce immunosuppression. aMPV-induced lesions in the upper respiratory tract affect the mucosal clearance and destroy the integrity of the epithelial layer. These irritations caused by aMPV may facilitate secondary bacterial infections. Infections with aMPV may also reduce vaccination efficacy against other pathogens. Thus, the most effective way to prevent TRT and the losses associated with subsequent disorders is to protect birds against aMPV infection.

Today protection against aMPV infection is achieved by live and inactivated vaccines. But these vaccines often induce only short-lived immunity. Additionally, live vaccines may display residual immunosuppressive and pathogenic effects with its consequences. Live attenuated vaccines also display the potential risk of reversion to virulence. New vaccination strategies need to be explored to overcome problems associated with current aMPV vaccines. New vaccination strategies should combine

the following cardinal characteristics: easy, save and cost effective production and the potential of climate-independent storage and application. Furthermore future aMPV vaccines should not have residual immunosuppressive or pathogenic effects. They should efficiently induce local protective immunity at the site of viral entry and replication. For this purpose genetically engineered vaccines may be promising candidates.

Some genetically engineered vaccines are already licensed for commercial use, such as a canarypox vector-based vaccine against West Nile Virus infection of horses (RECOMBITEK©, Merial) and a recombinant fowlpox-avian influenza vaccine for poultry (MICKLE et al. 1997). For humans plasmid-DNA vector vaccines already are under evaluation in clinical trials, for instance vaccines against Human Immunodeficiency Virus infection (ROBINSON 2007). For many other human or animal infectious pathogens vaccination strategies based on live viral vector vaccines, live recombinant bacterial vector vaccines, plasmid DNA vector vaccines or recombinant protein vaccines are under experimental development. Although live viral vectors, such as fowlpox virus and turkey herpesvirus, were shown to induce protection (FUCHS et al. 2006), their use in poultry production is problematic due to high prevalence of immunity against the most viral vectors used (SHARMA 1999). Plasmid DNA vectors that encode for immunogenic proteins of respective pathogens and recombinant proteins were also shown to induce specific protection, but in most studies the vaccines were given parenterally. This route is not efficient for economic application of vaccines in large poultry operations. Additionally, the genetically engineered vaccines are sensitive to enzymatic degradation *in vivo*. A delivery system is needed that allows mass application of these genetically engineered vaccines. The delivery system should protect these sensitive inocula against degradation *in vivo*. Poly ($_{D,L}$-Lactic-co-Glycolic Acid) (PLGA) microparticles (MP) meet these requirements. PLGA-MP are biocompatible microscopic capsules that efficiently protect the encapsulated or bound sensitive plasmid-DNA or recombinant protein vaccines against degradation *in vivo*. They are suitable for economic mass production and application, and can be administered by all possible routes.

Additionally PLGA-MP were shown to have powerful adjuvans-effects, such as induction of non-specific immune reactions and stimulation of MP-uptake by phagocytic cells.

For the development of new vaccination strategies knowledge about the immunopathogenesis of the pathogen of interest is essential. The importance of humoral and cellular, local and systemic immune reactions during infection and their contribution to protective immunity needs to be known. It has been shown that for aMPV it is important to specifically induce local cellular immunity in the upper respiratory tract at the site of viral entry and propagation.

The goal of this project was to develop a novel vaccination strategy against aMPV-infection in turkeys. The following two objectives were addressed:

1. To understand the immunopathogenesis of aMPV-A and aMPV-B in turkeys with emphasis on local cellular immune reactions.
   - The pathogenesis of infections with virulent and attenuated vaccine strains and also the pathogenesis of infections with subtypes A and B were compared.
   - A challenge model for the evaluation of local and systemic, humoral and cellular immune reactions to aMVP infection was established.
   - Application of this defined challenge model for the testing of the inductive and protective effect of a new vaccination strategy.
2. To develop a prime-boost vaccination strategy against aMPV-infection in turkeys, based on genetically engineered vaccines combined with PLGA-MP.
   - A plasmid-DNA-vaccine against aMPV was produced by insertion of the gene sequence encoding for the aMPV-A Fusion (F) protein into an eukaryotic expression vector.
   - F-specific DNA vaccine was evaluated for expression and immunogenicity *in vitro* and *in vivo*.

- A recombinant aMPV-A F protein was evolved homologue to the plasmid-encoded protein.
- The recombinant F protein was characterized *in vitro*.
- Protocols for the preparation of PLGA-MP were established.
- MP-based, genetic engineered vaccines were produced by adsorption of the plasmid-DNA to cationic MP and by encapsulation of recombinant F protein into MP.
- The MP-based vaccines were tested for safety and efficacy *in vitro*.
- The MP-based vaccines were tested in a DNA vaccine-prime and recombinant F protein-boost regime for safety, efficacy and protective power against challenge with virulent aMPV *in vivo*.

2. Literature review

2.1. Avian Metapneumovirus (aMPV) infection of turkeys

The avian Metapneumovirus (aMPV) is the causative agent of Avian Rhinotracheitis (ART), also named Turkey Rhinotracheitis (TRT) in turkeys and Swollen Head Syndrome (SHS) in chickens.

2.1.1. Etiology

aMPV is a member of the family *Paramyxoviridae* and of the subfamily *Pneumovirinae*, which consists of the genus *Pneumovirus* (including the human and bovine Respiratory Syncytial Viruses) and the genus *Metapneumovirus.* Currently the genus *Metapneumovirus* comprises avian Metapneumoviruses and human Metapneumoviruses.

All *Paramyxoviridae* are enveloped viruses that are encoded by non-segmented, single-stranded and negative-sense RNA. In contrast to other members of the family *Paramyxoviridae* aMPV does not display any neuraminidase or haemagglutinin activity. No haemagglutination was shown with erythrocytes of a variety of avian and mammalian species (WYETH et al. 1986). The virus is sensitive to heat and disinfectants, but is active in a pH-range of 3.0 to 9.0 (COLLINS et al. 1986; HAFEZ 1992; TOWNSEND et al. 2000). The viral particles are characterized morphologically by highly pleomorphic, fringed, spherical to filamentous structures. The particles are roughly 70-600 nm in diameter or length with a lipid-containing envelope that is displaying spikes (GIRAUD et al. 1986a; MCDOUGALL u. COOK 1986; COLLINS u. GOUGH 1988; ALEXANDER 1990; HAFEZ u. WEILAND 1990; HAFEZ 1991; O'LOAN et al. 1992).

The virus was extensively characterized by molecular sequencing (CAVANAGH u. BARRETT 1988). Gene sequences are published for all aMPV proteins, which are the following:

- The fusion (F) protein, which is expressed as a precursor protein called F_O that is cleaved to products F_1 and F_2 (YU et al. 1991; NAYLOR et al. 1998; BAYON-AUBOYER et al. 2000; SEAL et al. 2000)
- The attachment glycoprotein (G) (LING et al. 1992; JUHASZ u. EASTON 1994; BAYON-AUBOYER et al. 2000; ALVAREZ et al. 2003)
- The large RNA-dependent RNA polymerase (L) (RANDHAWA et al. 1996b; BAYON-AUBOYER et al. 2000; GOVINDARAJAN u. SAMAL 2004)
- The matrix and second matrix protein (M & M_2) (YU et al. 1992a; YU et al. 1992b; RANDHAWA et al. 1996a; SEAL 1998; DAR et al. 2003)
- The nucleocapsid protein (N) (LI et al. 1996; DAR et al. 2001)
- The phosphoprotein (P) (LING et al. 1995; DAR et al. 2001)
- The small hydrophobic protein (SH) (LING et al. 1992; YUNUS et al. 2003).

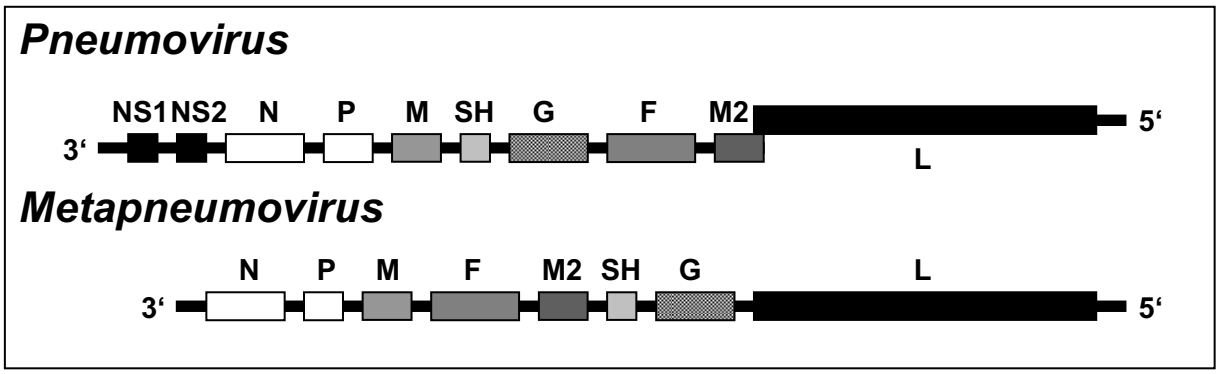

Fig. 2.1

Genome organization of members of the subfamily Pneumovirinae, modified scheme from Easton et al. (2004):

Genus *Pneumovirus*: Respiratory Syncytial Virus.

Genus *Metapneumovirus*: avian Metapneumovirus and human Metapneumovirus.

The aMPV-genome codes for the same set of viral proteins as the members of the genus *Pneumovirus* do, except for the absence of genes coding for the non-structural

proteins 1 and 2 (NS_1 & NS_2) in aMPV (RANDHAWA et al. 1997). Due to this aMPV has a genome of approximately 13.3 kb in contrast to the members of the genus *Pneumovirus,* which have a genome of approximately 15 kb .The gene order of the encoded proteins in the genus *Metapneumovirus* differs from the genus *Pneumovirus* (LING et al. 1992; YU et al. 1992b), as shown in Fig. 2.1.
The low aa sequence homology of aMPV to viruses from the genus *Pneumovirus* of about 40 % finally justified the definition of the new genus *Metapneumovirus* (PRINGLE 1999).

In 2001 the first human Metapneumovirus (hMPV) was isolated and classified as a member of the genus *Metapneumovirus (VAN DEN HOOGEN et al. 2001)*. Complete genome sequencing confirmed that the genomic organization of hMPV is similar to aMPV, and that at least one strain of hMPV is most closely related to aMPV subtype C (VAN DEN HOOGEN et al. 2002). The close relationship between hMPV and aMPV subtype C lead to the hypothesis that these viruses may be able to cross-infect (VAN DEN HOOGEN et al. 2001). This hypothesis was confirmed for infection of turkey poults with hMPV (VELAYUDHAN et al. 2006).

Isolates of aMPV are grouped to subtypes A to D. Field isolates of aMPV were classified as aMPV on the basis of electrophoretic analysis of viral proteins and mRNA (CAVANAGH u. BARRETT 1988; COLLINS u. GOUGH 1988), of serological analysis of antigenic relationships (COOK et al. 1993c; OBI et al. 1997; TOQUIN et al. 2000) and later on the basis of sequence analysis of genes and deduced aa sequences (JUHASZ u. EASTON 1994; JACOBS et al. 2003). The G protein sequence was used to subtype European strains. This was possible due to the high sequence identity of up to 99 % of isolates within the same subtype A or B in contrast to sequence identities of only up to 56 % between the subtypes A or B (JUHASZ u. EASTON 1994). The aa sequence of the G protein was used to classify the non-A and non-B French isolates as subtype D and the new emerging U.S. American isolates as subtype C (BAYON-AUBOYER et al. 2000; ALVAREZ et al. 2003). Nevertheless, based on phylogenetic analysis of F protein sequences SHIN et al.

(2002a) suggested that the European subtypes A, B and D are more closely related to each other than either of them is related to subtype C. More recently aMPV subtype C isolates were also identified from pheasants in Korea (LEE et al. 2007) and from ducks in France (TOQUIN et al. 2006). The latter, formerly reported as French non-A and non-B strains, were shown to be of a different genetic lineage than the U.S. American isolates. Irrespective of the differences within subtype C isolates this subtype displays a higher aa sequence homology to hMPV than to European aMPV subtypes A, B and D (VAN DEN HOOGEN et al. 2001; TOQUIN et al. 2003).

The principle mechanisms of aMPV replication is comparable to all other members of the family *Paramyxoviridae.* Not all target cells of aMPV have been identified yet. But cells of the upper respiratory tract, such as ciliated and non-ciliated epithelial cells of nasal turbinates, trachea and lung, are known to be target cells (MAJO et al. 1995; MAJO et al. 1996). Macrophages are suspected to be involved in viral spreading, because the viral genome has been detected in peripheral locations such as spleen cells (SHARMA et al. 2004b). To initialize replication the viral particle gets attached to the cell surface with its attachment G proteins. The F proteins induce fusion of the viral envelope to the cell surface membrane. The viral replication occurs in the cytoplasm, this may be independent of host nuclear mechanisms. The aMPV genome is transcribed by the viral RNA-dependent RNA polymerase into complementary mRNA of 8 transcriptional sequences, each coding for one viral protein or protein precursor. The replication of the viral RNA itself is based on an intermediate non-segmented complete positive-sense antigenome. The nucleocapsid is assembled in the cytoplasm at the location of RNA synthesis. The envelope is added during budding at the cell surface membrane at sites containing the essential viral envelope proteins.

2.1.2. Turkey Rhinotracheitis (TRT)

aMPV induces an acute, highly contagious infection of the upper respiratory tract of turkeys. Although young fattening turkeys between 4 – 9 weeks of age are most severely affected (ANDRAL et al. 1985b), the disease can affect all age groups. Adult

turkeys and laying parental flocks are less susceptible for the disease. The upper respiratory tract is predominantly affected in fattening turkeys, while in laying hens only a mild respiratory infection with a drop in egg production has been observed (JONES et al. 1988). Typical respiratory clinical signs in young fattening turkeys are: serous, watery nasal and ocular discharge; frothy eyes and conjunctivitis; at a later stage mucopurulent, turbid nasal discharge and plugged nostrils; swollen infraorbital sinuses; snicking, sneezing, coughing or tracheal rales (POLLAN 1992; PANIGRAHY et al. 2000; JIRJIS et al. 2002; PATNAYAK et al. 2002). These respiratory signs are accompanied by depression, anorexia and ruffled feathers.

The incubation period is three to seven days (JONES et al. 1987). In infected flocks the virus may spread rapidly through the entire flock within 12 to 24 hours (STUART 1989) always leading to high morbidity up to 100 %. The mortality may vary between 1 % and 30 % depending on age and constitution of the flock and on secondary infections. In case of non-controlled, severe secondary bacterial infections up to 90 % may decease (ALEXANDER 1990). Birds without secondary infections with good constitution or under experimental conditions may rapidly recover within 7 to 10 days post infection (PI) (VAN DE ZANDE et al. 1999). But with secondary infections and under poor management and hygiene regime the disease may be prolonged and exacerbated by airsacculitis, pericarditis, pneumonia and perihepatitis.

2.1.3. Transmission and epizootiology

Within or between turkey flocks the virus may spread horizontally by direct contact or by contact with contaminated objects (GIRAUD et al. 1986b; COOK et al. 1991). The virus was found to rapidly spread within and between turkey flocks and thus was assumed to be highly contagious (DAR et al. 2002; NAYLOR et al. 2002). Otherwise the virus is rapidly destroyed due to its enveloped nature after its release from the host to the environment (JONES 1996). As aMPV affects the upper respiratory tract, transmission is most likely to be airborne (GIRAUD et al. 1986b), especially by aerosol (ALEXANDER 1990).

aMPV subtype C was isolated from eggs of experimentally infected SPF laying turkey hens up to 7 days PI (KAPCZYNSKI 2005). But JONES et al. (JONES et al. 1988) suggested that the vertical route may be short-lived and may play only a minor role in aMPV transmission.

Numerous studies with respect to re-isolation of aMPV after challenge have shown that the birds shed aMPV only for a few days PI. This short period of shedding suggests that there is no latency or carrier status of birds (COOK et al. 1993b; KHEHRA 1998; VAN DE ZANDE et al. 1999). Reconvalescent flocks can repeatedly be re-infected with aMPV within one fattening period (REDMANN et al. 1991). Due to that permanent circulation of aMPV within a flock, a farm or region of poultry production may occur. On the other hand BUYS et al. (1989a) showed that reconvalescent birds are refractory to aMPV re-infection for up to 6 weeks following natural outbreaks.

The spread of aMPV appeared to depend on the poultry population density, standard of hygiene and biosecurity. Additionally there seems to be evidence that migratory birds could have been involved in initial spread from southern African to European countries (STUART 1989). More recently it was discussed whether aMPV in turkeys in USA might have originated from wild bird populations (SHIN et al. 2000; BENNETT et al. 2004).
aMPV may not only replicate in turkeys, but also in chickens, pheasants, Muscovy ducks and guinea-fowl (GOUGH et al. 1988; TOQUIN et al. 1999). GOUGH et al. (1988) demonstrated that geese, pigeons and most ducks seemed to be refractory to disease. SHIN et al. (2000) detected aMPV subtype C RNA in geese, sparrows and starlings that were sampled in regions of aMPV outbreaks in turkey flocks in Minnesota. RNA from aMPV was also detected in house sparrows, Canada geese, blue-winged teal and ring-billed gulls sampled in Minnesota, and in snow geese from Saskatchewan, Canada. These findings provide evidence for the circulation of aMPV in wild birds that are not always in direct contact to poultry farms (BENNETT et al. 2002; BENNETT et al. 2004).

2.1.4. Geographical distribution, prevalence and incidence

BUYS & DuPREEZ (1980) were the first to describe an acute infection in the upper respiratory tract of turkeys in South Africa in 1978. Since then aMPV-infections in chickens or turkeys were described in France (ANDRAL et al. 1985a), in the UK (O'BRIEN 1985; WILDING et al. 1986), and subsequently in other European countries (GOREN 1985; HAFEZ 1987; COOK et al. 1993a; NAYLOR u. JONES 1993). Up to now aMPV was reported in Israel (WEISMAN et al. 1988), Canada (ZELLEN 1988), Japan (URAMOTO et al. 1990), Morocco (WYETH 1990), Mexico (DECANINI et al. 1991), Brazil (ARNS u. HAFEZ 1992), Central America (JONES 1996) and most recently in the USA (SENNE et al. 1997; COOK et al. 1999) and Korea (LEE et al. 2007). The absence of antibodies to aMPV was recorded in Australia (HECKERT u. MYERS 1993) and Canada (HECKERT u. MYERS 1993). But more recent aMPV RNA was detected in Canadian wild birds (BENNETT et al. 2004). This suggests an almost global distribution of aMPV, at least in countries conducting industrial poultry production, except for Australia (COOK 2000).

The incidence of new aMPV-infections is very high when aMPV spreads to naïve poultry populations. This was recorded for the initial outbreaks in the UK and in Minnesota (STUART 1989). Interestingly, the first aMPV epizootic in Colorado, which affected only a small turkey population, was controlled in less than one year after the outbreak by means of slaughter and biosecurity (SENNE et al. 1997; COOK et al. 1999). In contrast to that, in the UK and in Minnesota the virus has been circulating for several years with high incidence and prevalence. Although the aMPV-prevalence decreased due to advances in awareness, management and control measures, aMPV is still present in many countries (GOYAL et al. 2003). The latter study revealed that the seroprevalence displayed a seasonal bias with peak incidences in spring and fall. It also revealed that the rate of seropositive flocks correlated with regional density of turkey flocks. A high prevalence of aMPV in poultry producing areas was reported for several countries worldwide, such as for Germany (HAFEZ 1990), Japan (OTSUKI et al. 1996) and Israel (BANET-NOACH et al. 2005). A serosurvey study in ostriches sampled in Zimbabwe revealed 99 % seropositive birds

(CADMAN et al. 1994). We may assume that aMPV circulates in almost all fattening turkey flocks in Germany. Studies on the seroprevalence of aMPV-specific antibodies in chickens were conducted in many countries (WYETH et al. 1987; HAFEZ u. LOHREN 1990; OTSUKI et al. 1996; D'ARCE et al. 2005; OWOADE et al. 2006; GHARAIBEH u. ALGHARAIBEH 2007). In all studies a high prevalence of aMPV-specific antibodies was demonstrated, irrespective of the current occurrence of clinical swollen head syndrome.

For the interpretation of surveillance data that were obtained by ELISAs or other serological tests, it is important to take into account the subtype-specifity of the test systems. The subtype-specifity of the applied test may result in limited or no detection of other subtypes or new emerging aMPV strains that do not cross-react.

2.1.5. Macroscopic and microscopic lesions following aMPV infection

The macroscopic lesions found after aMPV infection largely depend on the course of infection, especially on secondary bacterial infections (VAN DE ZANDE et al. 1998; JIRJIS et al. 2004). Gross lesions induced in experimental aMPV-infections under controlled environmental conditions are due to rhinitis, tracheitis, sinusitis and aersacculitis (NAYLOR et al. 1992; MARIEN et al. 2005). But also no gross lesions may be observed in aMPV infected turkeys (COOK et al. 1993b). Serous to turbid mucous may be observed in the nasal cavity, nasal turbinates, trachea and in infraorbital sinuses. During the course of infection the secreted mucous turns from clear and serous to turbid and purulent. Also non-specific signs of inflammation, such as swelling and hyperemia of the mucosa and excessive mucous (VAN DE ZANDE et al. 2001a), can be seen in the upper respiratory tract and in air sacs. The macroscopic lesions are most prominent on days 4 to 10 PI. If the infection is exacerbated by secondary bacterial infections, copious inflammatory exsudate is found in the respiratory tract, but also pneumonia, pericarditis, perihepatitis and spleno- and hepatomegaly were observed (COOK et al. 1991). In the reproductive tract of laying turkeys various lesions were observed, such as egg peritonitis, ovary

and oviduct regression, folded shell membranes in the oviduct and misshapen eggs were found (JONES et al. 1988).

The microscopic lesions induced by aMPV infection were extensively examined by Majó et al. (MAJO et al. 1995) and also by further authors (JONES et al. 1986; VAN DE ZANDE et al. 1999; MARIEN et al. 2005; AUNG et al. 2008). Histopathological changes were observed at first and were most pronounced in the mucosa of the nasal turbinates. At 1 to 2 days post aMPV infection increased glandular activity was observed with glandular hyperplasia, focal loss of epithelial cilia or even the epithelial layer. Also congestion, distension and infiltration of the mucosa and submucosa with immune cells was seen. Between 3 to 5 days PI the predominant lesions were the damage of the epithelial layer and a copious infiltration of the submucosa with immune cells. In detail, the following findings were described:

- Extensive loss of epithelial cilia.
- Irregular shape of the mucosa due to necrosis of epithelial cells, vacuolation and accumulation of cell debris within the epithelial layer, obliteration of the mucous glands, congestion or hyperemia and thickening of the mucosa.
- Diffuse sub- and intraepithelial as well as submucosal mononuclear, lymphocytic and heterophilic infiltration.
- In more dramatic cases complete necrosis and hemorrhage of the epithelial layer, fibrin accumulation and substantial submucosal heterophilic infiltration.

These acute effects of infection were even more advanced at 6 to 10 days PI. In the later stage of infection they were displaced by the unspecific signs of epithelial repair, such as metaplasia of the regenerating epithelium. Transient lesions were found to occur predominantly in the nasal turbinates and less pronounced in the trachea. Other tissues like conjunctiva, air sacs and lung were virtually non-affected. The nasal turbinates were suggested to be suitable for microscopic evaluation and diagnostic of aMPV infections (MAJO et al. 1995).

2.1.6. Public health implications

In 2001 the first isolate of human Metapneumovirus (hMPV) was characterized and classified as a member of the genus *Metapneumovirus* (VAN DEN HOOGEN et al. 2001). In a retrospective study, the same author stated that hMPV circulated among humans in the Netherlands at least since 1958. High incidence of hMPV among humans was also reported for Australia (NISSEN et al. 2002) and Canada (PERET et al. 2002). Based on these reports, hMPV is suggested to be widely distributed, and subsequent retrospective studies revealed the involvement of hMPV in a variety of respiratory infections of previously unclear aetiology (BROOR u. BHARAJ 2007).

The comparison of aMPV of all subtypes with hMPV revealed that the highest aa identity of viral proteins of 80 % was detected between aMPV subtype C and the examined strain of hMPV. The overall percentage of sequence identity between individual proteins of hMPV and aMPV subtype C was sometimes higher than the percentage observed within all four subtypes of aMPV (SHIN et al. 2002b; VAN DEN HOOGEN et al. 2002).

Because of the newly discovered clinical importance of hMPV infections, especially in children younger than one year old, it may be necessary to clarify the link between hMPV and aMPV-C. Only little work was published on this topic so far.
In preliminary studies macaques were susceptible to hMPV infection and virus replication *in vivo* (VAN DEN HOOGEN et al. 2001). Also turkey poults were shown to be susceptible to hMPV infection (VELAYUDHAN et al. 2006). This was in contrast to no susceptibility of juvenile chickens or turkey poults to hMPV infection or virus replication in the aforementioned study (VAN DEN HOOGEN et al. 2001).
Further informations on cross-infections between avian species and humans are lacking (BROOR u. BHARAJ 2007). The relatively low aa identity between the glycoproteins of hMPV and aMPV, which are responsible for attachment and thus for the cell tropism, may be a reason for low or even no cross-infection capability due to restricted host range.

2.1.7. Consequences of aMPV field infections

The secondary effects of aMPV infection, such as reduced, inhomogeneous weight gain, poor feed conversion, increased mortality and condemnation of carcasses, are the major causes for losses during production and slaughter. These effects are the consequences of the impairment of the respiratory mucosal barrier. Also the generally depressed immune reactivity due to the immunosuppressive effect of aMPV infection and environmental stress may contribute to those adverse consequences. In almost all cases those factors support secondary bacterial infections (HAFEZ 1994; VAN DE ZANDE et al. 1998). The enhancement of bacterial infections by foregoing, parallel or subsequent aMPV infection was demonstrated for a variety of bacterial avian pathogens, such as *Bordetella avium* (COOK et al. 1991; JIRJIS et al. 2004), *Pasteurella*-like organisms (COOK et al. 1991), *Escherichia coli* (VAN DE ZANDE et al. 2001a; TURPIN et al. 2002; VAN DE ZANDE et al. 2002; JIRJIS et al. 2004), *Ornithobacterium rhinotracheale* (JIRJIS et al. 2004; MARIEN et al. 2005), *Chlamydophila psittaci* (M. VAN LOOCK et al. 2002; A. VAN LOOCK et al. 2006), *Mycoplasma gallisepticum* (NAYLOR et al. 1992) and *Mycoplasma synoviae* (KHEHRA et al. 1998). Also the synergistic effects with additional viral avian pathogens were evalutated for aMPV infections in combination with Newcastle Disease virus (GANAPATHY et al. 2005), turkey Herpesvirus (VAN DE ZANDE et al. 2001b) and Hemorrhagic Enteritis virus (CHARY et al. 2002c).

2.1.8. Pathogenesis

A variety of experimental challenge studies or field studies were done to evaluate the pathogenesis of aMPV-infections. The pathogenesis is characterized by a variety of parameters like clinical outcome, macroscopic and microscopic lesions, viral shedding and humoral immune response. More recently, also studies with the focus on the CMI to aMPV-C were conducted.

Studies on microscopic lesions induced by aMPV infection and also on viral isolation or detection of aMPV-genome in affected tissues revealed that the cell and tissue

tropism may be limited to the epithelial layer in the upper respiratory tract. In layers and breeders the reproductive tract may be affected additionally (MAJO et al. 1995; MAJO et al. 1996; VAN DE ZANDE et al. 1999). In industrial poultry production the virus most likely may spread to the respiratory mucosal surface via aerosol or dust particles. Once the virus is on the epithelial layer, the attachment G protein mediates the link of the viral particle to the epithelial cells membrane (LEVINE et al. 1987). Subsequently the F protein induces the fusion of the viral envelope with the host's cell membrane. Finally the viral genome enters the cytoplasm and virus propagation proceeds irrespective of the nucleus (FOLLETT et al. 1975). Rapid propagation and virus shedding enhances the spread of aMPV within the mucosa of the upper respiratory tract of the affected bird, but also within the affected turkey flock. Sharma (2004b) demonstrated that aMPV-genome can be detected systemically in the blood circulation and in spleen tissue following aMPV-C infection of turkeys. He considered macrophages, which were shown to be susceptible to aMPV infection *in vitro*, to be involved in systemic spread of aMPV.

The clinical disease of aMPV-infection was seen between days 2 to 10 PI, with the highest incidence and severity of clinical signs between days 5 to 7 PI (MCDOUGALL u. COOK 1986; COOK et al. 1993b; NAYLOR u. JONES 1994; VAN DE ZANDE et al. 1999; JIRJIS et al. 2002; MARIEN et al. 2005), please also refer to chapter 2.1.2. The clinical disease appeared to coincide with viral shedding. In field samples shedded virus was isolated from day one to day seven PI, and in experimental studies up to 10 days PI (VAN DE ZANDE et al. 1999). In chicken, viral genome even was detected up to 28 days PI in choanal swabs (HESS et al. 2004b). Based on those studies, it may be concluded that the duration of viral shedding is limited and that no carrier or latent status occurred. Additionally, it may be speculated that the presence and clearance of aMPV in the mucosa correlates with the outcome and recovery of microscopic lesions, respectively (COOK et al. 1993b; MAJO et al. 1995; AUNG et al. 2008). Please refer also to chapter 2.1.5.

Timms et al. (1986) were the first to demonstrate an immunosuppressive effect of aMPV. The more the birds of the aMPV field-challenged groups were clinical affected, the more the parameters of CMI, such as wing web intradermal phytohaemagglutinin response and relative thymus weight, were depressed.

With the occurrence of aMPV-C in the USA the research on the pathogenesis of aMPV infections was reopened. The immunosuppressive effect that was discussed for aMPV-A and -B was experimentally confirmed for aMPV-C (SHARMA et al. 2000; CHARY et al. 2002b; CHARY et al. 2002c; TURPIN et al. 2002). Chary (2002b) demonstrated that aMPV-C may induce inhibition of T cell responsiveness in the acute phase of infection. At 3 and 5 days post aMPV-C infection, spleen cells of infected birds showed markedly reduced proliferative response to concanavalin A (Con A). As with aMPV-A and aMPV-B before, no significant influence of humoral immune reactions on the pathogenesis of aMPV-C infection was demonstrated. aMPV-C infection had no effect on the antibody-producing ability of turkeys against several extraneous antigens such as *Brucella abortus* and tetanus toxoid (CHARY et al. 2002b).

2.1.9. Immune reactions to aMPV

The immune reactions during aMPV infection were shown to predominantly be characterized by the importance of cell mediated immunity (CMI) in the control of the infection. In contrast to that the humoral immunity was considered to not play a significant role in protection (Fig. 2.2).

The humoral immune response to aMPV infection was examined in a variety of studies (JONES et al. 1987; JONES et al. 1988; COOK et al. 1991; COOK et al. 1993b; VAN DE ZANDE et al. 1999). Field infections induced production of significant titres of VN antibodies as early as 5 days PI, and of ELISA antibodies as soon as 7 days PI (JIRJIS et al. 2002). The most substantial experimental work on humoral immune responses to aMPV-A vaccination and subsequent challenge with

virulent aMPV-A was done by Khehra (1998). He specifically detected IgA and IgG antibodies in lacrimal fluid, tracheal washes and serum following vaccination and challenge. The observed early local B cell response, particularly of IgA+ B cells in the mucosa of the respiratory tract, was confirmed for (re-)infections with aMPV-C (CHA et al. 2006). In the latter study also infiltration of the mucosal layer by IgG+ and IgM+ B cells was demonstrated.

Jones et al. (1992) applied cyclophosphamide treatment to turkeys before aMPV vaccination and challenge. This treatment is known to induce a severe permanent deficiency in the humoral response but only a short-lived depression of the thymic system. The treatment had no effect on the resistance to aMPV challenge following vaccination. It was assumed that the protection against aMPV challenge is independent of humoral immunity.

It was shown that aMPV-C infections stimulated the accumulation of local CD4+ T cell subpopulations and the production of CMI related cytokines (CHARY et al. 2002a; SHARMA u. GERBYSHAK-SZUDY 2002; SHARMA et al. 2004b). Increased levels of IFNγ transcript levels were detected in the HG as well as in the spleen up to 7 days post aMPV-C infection by real-time RT-PCR (SHARMA u. GERBYSHAK-SZUDY 2002).

Ex vivo stimulation of spleen cells with Con A and lipopolysaccharide stimulated spleen cells from infected birds to release nitric oxide-inducing factors (NOIF) in contrast to no release of NOIF by spleen cells of control birds. With these observations the importance of CMI in protection against aMPV infection was supported (CHARY et al. 2002b). But it may also concluded that the cellular immune reactions are involved in the induction of lesions.

Only few studies were done on the immune reactions to aMPV-A and aMPV-B (KHEHRA 1998). In particular almost no work was done on the role of CMI in aMPV-A and aMPV-B infections of turkeys.

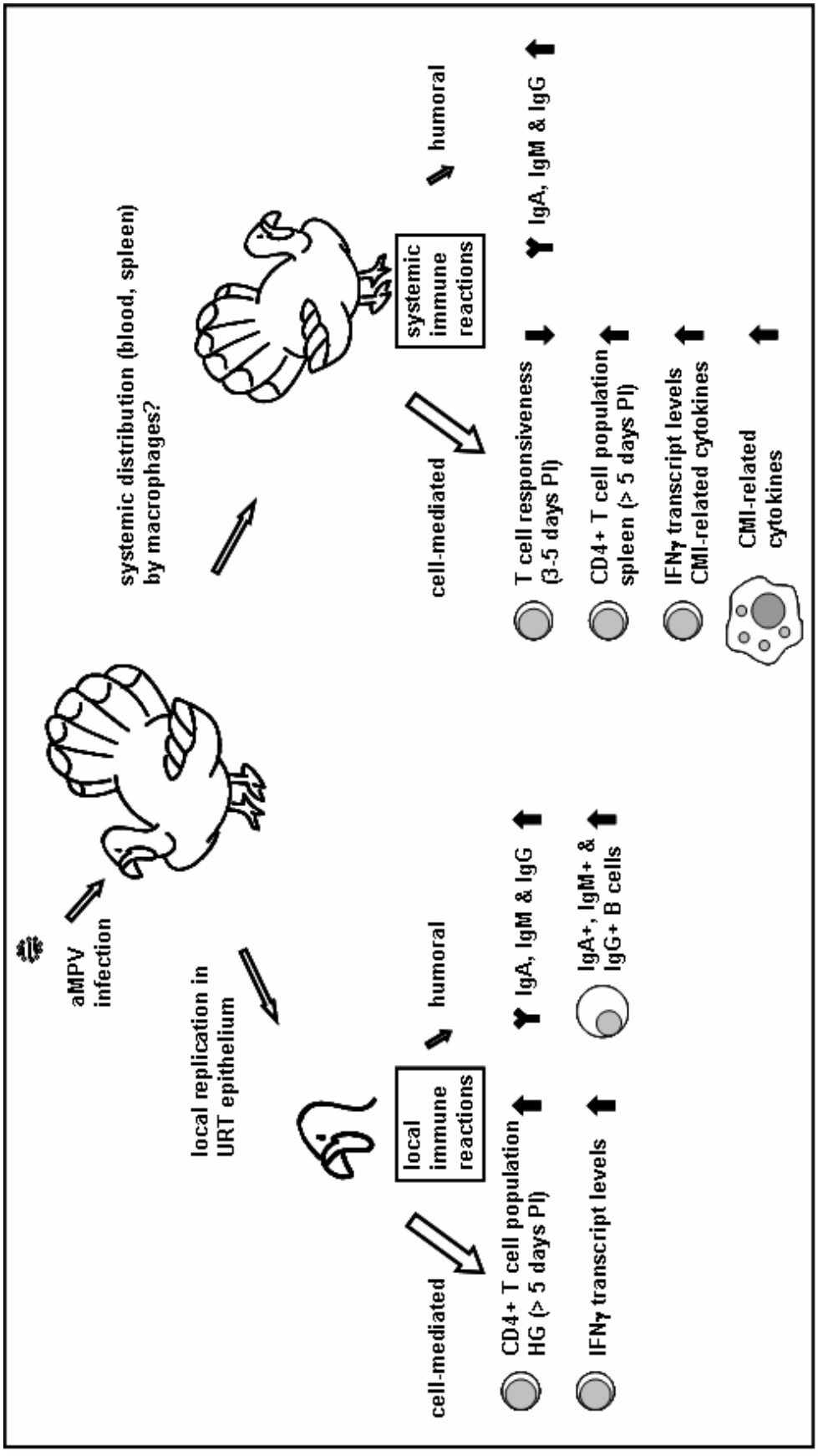

Fig. 2.2 Model for the aMPV-pathogenesis and the immune reactions in turkeys.

2.1.10. Diagnosis

Initially, diagnosis of aMPV infection was difficult due to problems in isolation of the aetiological agent. It also was hindered by the high frequency of parallel isolation of further bacterial and viral pathogens, which may have misled the aetiological conclusions. These days, the consideration of the syndrome character of aMPV field infections and the directed use of a set of established diagnostic tools support the diagnosis of aMPV.

2.1.10.1. Clinical signs and lesions

Diagnosis of aMPV infection can not be based solely on clinical signs. Only in aMPV-naïve flocks under good management with low infectious pressure of bacterial pathogens and optimized ventilation, the early stage of a primary clinical aMPV infection may be diagnosed. In most cases the secondary infections dominate the clinical signs. Please refer also to chapter 2.1.2.

The macroscopic and microscopic lesions induced by aMPV infections (please refer to chapter 2.1.5) can not be differentiated from those seen in infections of the upper respiratory tract with a variety of other avian bacterial and viral pathogens.

2.1.10.2. Virus isolation

Extensive work has to be done on the isolation of unknown strains of aMPV. Subtype, host range and pathogenicity of the isolates may differ. This needs to be considered in the approaches chosen for isolation (GOUGH et al. 1998b; COOK 2000).

Primary aMPV isolation

The European strains of subtype A and B can be successfully isolated in two host systems:

- Six- to eight-day old embryonated chicken or turkey eggs from aMPV-negative flocks are inoculated via the yolk sac route (BUYS et al. 1989a). aMPV may

cause haemorrhages and embryo mortality. But usually a series of at least two to three passages of homogenized allantoic fluid and yolk sac membrane is required for consistent outcomes. This first method is expensive and time consuming. But it is the method of choice for strains that do not cause ciliostasis and therefore are not suitable for the second method using tracheal organ cultures (TOC). The strains of American subtype aMPV-C do not induce ciliostasis and thus were initially isolated by this first method (COOK et al. 1999).

- TOC are prepared from tracheal rings of chicken or turkey embryos harvested shortly before hatching or from one- to two-day-old birds (MCDOUGALL u. COOK 1986; WILDING et al. 1986; WYETH et al. 1986). After inoculation strains of aMPV subtype A and B cause ciliostasis at six to ten days PI. The virus titre peak is reached at three to five days post inoculation (COOK et al. 1991). Thus, every three to four days PI a blind passage with culture supernatant is done with a portion of replicates. The remaining replicates are observed for ciliostasis up to ten days PI.

Following primary isolation of a new aMPV strain with one of these two methods, the strain has to be confirmed to be aMPV and, if desired, can be adapted to grow in cell cultures. Confirmation of aMPV isolation in embryonated eggs or in TOC was done by one of the methods described in chapter 2.1.10.3.

In vitro adaptation of aMPV to cell cultures

Primary avian cell cultures, namely chicken embryo fibroblasts (CEF) (GRANT et al. 1987) and chicken embryo liver cells (CEL) (WILLIAMS et al. 1991a), and also the mammalian VERO cell line (BUYS et al. 1989a; WILLIAMS et al. 1991a) can readily be used to adapt any aMPV field strain to the cell culture system.

A primary isolation of either subtype of aMPV can also be conducted in cell culture, such as in VERO cells. This method is even less laborious than isolation in the two host systems described above (TOQUIN et al. 2006; GUIONIE et al. 2007). But it must be taken into account that the field strain may increasingly be modified

(attenuated) beginning with the first passage in cell culture, when compared to passage *in vivo*.

Further considerations in aMPV isolation

Three additional factors have to be considered for isolation of aMPV, namely the choice and timing of sampling and the risk of isolation of vaccine strains. Virus can be isolated from trachea, lung and also from visceral organs, but the most promising samples are nasal exudates and scrapings of sinus tissue (GOUGH et al. 1998a). It is crucial to take samples at the very beginning of aMPV infection, as the virus can only be isolated between one to five days PI. As soon as clinical signs are obvious, the isolation of aMPV may not be successful anymore (JONES 1996).

Isolation of virulent field virus may be complicated by sole isolation of vaccine virus or isolation of a mixture of virulent field virus and vaccine virus. As the live vaccine strains are derived from virulent field strains by passage-attenuation, the differentiation between those strains may be achieved by molecular biological methods, i.e. hybridisation techniques or sequencing. Cavanagh (1997) succeeded in differentiating between an aMPV-B vaccine strain and its progenitor field strain. But this differentiation was based on only three aa changes in the entire genome, and the stability of these changes was not proven yet.

Based on the growing number of genome sequence data and the easy and priceless access to sequencing techniques, detailed characterization and molecular differentiation of isolated aMPV strains is more and more done (CHACON et al. 2007).

2.1.10.3. Virus detection

Virus detection is used in clinical veterinary diagnostic, for specific confirmation of aMPV isolation *in vitro* and for experimental approaches. Initially detection was conducted by electron microscopy (BUYS u. DU PREEZ 1980; GIRAUD et al. 1986b; MCDOUGALL u. COOK 1986), by immunolabelling techniques, such as

immunoperoxidase (O'LOAN u. ALLAN 1990; CATELLI et al. 1998), immunofluorescence (JONES et al. 1988) or immunogold (O'LOAN et al. 1992) techniques.

More recently, the reverse-transcriptase polymerase chain reaction (RT-PCR) provided a tool for specific detection of aMPV by RT-PCR or RT nested-PCR. These PCR techniques allow the detection of aMPV or the specific detection of subgroups aMPV-A to aMPV-D (JUHASZ u. EASTON 1994; BAYON-AUBOYER et al. 1999; BAYON-AUBOYER et al. 2000; TOQUIN et al. 2003). In addition to specific detection of aMPV this technique provides amplicons that can be sequenced for further phylogenetic characterization of the isolate. These days the PCR techniques are complemented by subtype-specific real-time RT-PCR assays (GUIONIE et al. 2007).

2.1.10.4. Serology

Extensive efforts have been made on the development of serological methods for detection of aMPV-specific antibodies. Although methods like virus neutralization testing (VNT) (BAXTER-JONES et al. 1989) and indirect immunofluorescence tests exist (BAXTER-JONES et al. 1986), the ELISA is the most commonly used serological method (GRANT et al. 1987; O'LOAN et al. 1989; ETERRADOSSI et al. 1995; OBI et al. 1997; GULATI et al. 2000; ALKAHALAF et al. 2002; TURPIN et al. 2003; ALVAREZ et al. 2004).

Independent of the applied test system, sera should be tested in pairs, including sera taken in the acute phase of infection as well as sera of reconvalescents.

Today a number of commercial aMPV-specific ELISA kits are available. Some of these kits afford the testing of multiple subtypes and the testing of sera of any avian species. Unfortunately, the kits greatly vary in subtype-specific sensitivity. This was demonstrated by Mekkes and de Witt (1998) by comparing three commercial aMPV-specific ELISA kits. In this study all kits revealed 100% specifity but varying sensitivity. This study supported those observations made by Eterradossi et al. (1995). He used in-house and commercial ELISA kits as well as VNT for the

detection of aMPV-specific antibodies following vaccination and/ or challenge with a range of different attenuated and virulent aMPV isolates. This study revealed that the inadequate choice of coating antigen may totally hinder or interfere with the detection of actually existing antibodies following vaccination or challenge infection. These problems should be considered when choosing a commercial kit or an in-house method. It may be benefitial to use homologous antigen for detection of antibodies of suspected subtype (TOQUIN et al. 1996; MEKKES u. DE WITT 1998; COOK et al. 1999).

Detection of aMPV-neutralizing antibodies is done by standard neutralization techniques. This technique is meaningful and adaptable to scientific approaches, but more time consuming and expensive than ELISA technique and for this reason less applicable for serological screening in the field. With respect to the subtype-specific application a variety of host systems, such as CEF, CEL, VERO cells or TOC can be used for VNT (GIRAUD et al. 1986b; O'LOAN et al. 1989; REDMANN et al. 1991; WILLIAMS et al. 1991a; COOK et al. 1993a; GOUGH et al. 1998b). Strain-dependent cross-reactivity between subtypes aMPV-A and -B were demonstrated by Hafez (1994). Baxter-Jones et al. (1989) showed a good correlation between VNT and ELISA as well as indirect immunofluorescence tests.

2.1.11. Control of aMPV

Although a variety of commercial aMPV vaccines is available, sustainable vaccination efforts and even more the eradication of aMPV remains a challenge to the poultry industry.

2.1.11.1. Vaccination

The severity of aMPV field infections can effectively be reduced by vaccination, as demonstrated in a variety of field and experimental studies (BUYS et al. 1989b; COOK et al. 1989a, 1989b; WILLIAMS et al. 1991a; WILLIAMS et al. 1991b; GULATI

et al. 2001; PATNAYAK et al. 2002; RAUTENSCHLEIN et al. 2002; PATNAYAK et al. 2003).

In the planning of a vaccination programme for the induction of a long-lasting protective immunity a few factors have to be taken into account. On one hand maternally derived antibodies do not provide protection against aMPV infection (COOK et al. 1989b; NAYLOR et al. 1997; VAN DE ZANDE et al. 2002). Thus, a vaccination programme should comprise the first immunization as soon as possible after hatching. On the other hand it is crucial to achieve a homogenous state of immunization per flock and farm. It is important to assure application of an adequate vaccine dose to all birds per flock and farm.

Live attenuated vaccines are used for the immunization of grow out turkeys and broilers, but also for the priming in the rearing period of layer and breeder flocks. Several commercial live attenuated vaccines have been licensed in many countries (COOK 1999). The recommendations of the suppliers for the interval of application vary between three and five weeks, and most of them recommend the prime immunization in the first week of age. Live attenuated vaccines were shown to induce local respiratory humoral immunity as well as systemic humoral immunity (KHEHRA 1998). Birds free of detectable aMPV-specific antibodies (COOK et al. 1989b) or even birds treated with cyclophosphamide (JONES et al. 1992) show at least partial protection against aMPV challenge infection. Based on these observations and on the studies on the cell mediated immune reactions to aMPV-C (CHARY et al. 2002a; SHARMA u. GERBYSHAK-SZUDY 2002; SHARMA et al. 2004b), it was speculated that CMI is the most important mediator of overall protective immunity to aMPV infection (please see also chapters 2.1.8 and 2.1.9). Live attenuated vaccines are considered to be more powerful than inactivated vaccines for induction of protective immunity. But there are some critical problems related to this vaccination strategy:

- It was shown that the protective effects of immunizations that are solely based on live attenuated vaccines were too short-lived, especially for the grow out of toms (VAN DE ZANDE et al. 2000; PATNAYAK u. GOYAL 2004, 2006).
- Thus, repeated re-vaccination of turkeys is common practice (COOK 2000).
- Despite vaccination field infections often occur (VAN DE ZANDE et al. 1998).
- Live vaccines may have some residual immunosuppressive abilities and thus may support secondary bacterial and viral infections (CHARY et al. 2002b; CHARY et al. 2002c).
- There is a risk of reversion to more virulent variants up to the virulence of the original strain initially used for attenuation (CATELLI et al. 2006).

Inactivated aMPV vaccines are used for booster immunization of layer and breeder flocks following priming with live vaccines. While inactivated vaccines alone induce only partial protection against aMPV infections, the most powerful and long-lasting protection is achieved by a combined prime-boost vaccination program. This program comprises repeated priming with live attenuated vaccines and booster immunization with inactivated oil-adjuvanted vaccines (COOK et al. 1996).

A range of experiments and field observations were made with respect to cross-protection between different aMPV subtypes (HAFEZ 1992, 1994; COOK et al. 1995; ETERRADOSSI et al. 1995; TOQUIN et al. 1996; COOK et al. 1999). Most distinctive cross-protection is found between aMPV-A and aMPV-B. But cross-protection is also found between American and European aMPV-C strains and between European aMPV-A and -B and American aMPV-C. In either case the degree of cross-protection varies and is not steadily predictable depending on the involved aMPV strains.

More recently some studies were conducted on the protective power of *in ovo*-vaccination against aMPV infections (WORTHINGTON et al. 2003; HESS et al. 2004a; TARPEY u. HUGGINS 2007). These studies revealed that the *in ovo*-vaccination may be a promising strategy for effective, early induction of an immune response. The onset of specific immunity against aMPV was found to be earlier and

the antibody titres were found to be higher in *in ovo*-vaccinated hatchlings in comparison to hatchlings vaccinated with the same live attenuated vaccine at day of hatch.

Besides live attenuated and classical inactivated vaccines some experimental, genetic engineered vaccines were designed and tested for its protective capability, these vaccines are introduced in chapter 2.2.

2.1.11.2. Treatment and eradication

No specific treatment of monocausal aMPV infections in turkeys is available. It is compulsory to take action with regard to management factors, as they greatly influence the outcome and severity of the clinical infection, mainly by supporting secondary infections (ANDRAL et al. 1985b). These management factors comprise ventilation, temperature control, stocking density, multi-age stocking and litter quality, but also general hygiene and systematic supplementation of feeding, e.g. by vitamins and/ or antioxidants. Secondary bacterial infections should be controlled by adequate antibiotic therapy, based on resistance testing (HAFEZ et al. 1990; HAFEZ 1994).

The importance of strict biosecurity and good management practices was demonstrated in the only documented case of definite eradication of aMPV-C in the State of Colorado, USA. Although the virus circulated in more than one production cycle, by means of strict biosecurity and good management practices aMPV-C was totally eradicated (COOK 2000).

2.2. Genetic engineered vaccines

2.2.1. Current vaccination strategies in poultry

Vaccination is a basic and essential tool, particularly in poultry production, to protect an organism against infection with a particular pathogen. The majority of economical important poultry is raised and grown out in concentrated areas with strong infectious pressure. Thus, vaccination is needed to protect against particular pathogens (SHARMA 1999).

For strategical and effective vaccination the epizoological status of the affected area has to be considered. Further factors, such as management and hygiene concerns, are as important as vaccination and strictly have to be considered.

2.2.1.1. Commonly used vaccines and application routes

Vaccines can be applied to poultry by various routes. Inactivated vaccines and also some strains of live viral vaccines are applied by the parenteral route. This route induces normally a strong and long lasting humoral immune response. But the effect of inactivated vaccines on the CMI-mediated protection at the musosal site is limited (ROTH 2002). The latter is effectively achieved by live vaccination via oral, intranasal or conjunctival route. The majority of vaccines in commercial poultry production are applied by these routes. The vaccines are applied by drinking water or by coarse spray (SHARMA 1999; ROTH 2002). The advantage of these two ways of application is that they are less laborious and thus more cost-effective in contrast to parenteral vaccination.

In poultry there is one additional route of vaccination. The *in ovo* vaccination was shown to not negatively affect any breeding or hatching parameter. But it effectively induces early onset of protection against the respective pathogen (SHARMA 1986; RICKS et al. 1999).

Passive immunization is rarely applied in poultry production.

2.2.2. New vaccination strategies

Besides the classical vaccine type a variety of new vaccination strategies have been considered for use in immunization against avian pathogens. These strategies comprise

a.) immune complex vaccines, e.g. for protection against infectious bursal disease (WHITFILL et al. 1995; JEURISSEN et al. 1998) (subcutaneous or *in ovo* application);

b.) conventional subunit vaccines, e.g. a virosome vaccine for protection against aMPV-C infection (KAPCZYNSKI 2004). This aMPV-C virosome vaccine was produced by detergent-based extraction of immunogenic proteins from whole virus particles, followed by resembling of the envelope structure including the major antigenic determinants, such as F and G protein.

c.) More recent experimental, new vaccination strategies against aMPV infection are designed by genetically engineering of vaccines. In the following chapters these genetically engineered vaccines types are introduced and discussed for their applicability in protective immunization against aMPV-infection.

2.2.2.1. Genetically engineered subunit-/ recombinant protein vaccines

Genetically engineered subunit or recombinant protein vaccines are designed by cloning of specified protein-coding sequences into *in vitro* expression systems. This is in contrast to classical engineered subunit vaccines. They are based on purification of native immunogenic proteins from whole virus particles. The most widely used *in vitro* expression systems are prokaryotic expression plasmid vectors introduced to E. coli strains. Proteins purified from these systems are capable of inducing protective immunity following parenteral or oronasal application.

For mammalian pneumoviruses the immunogenicity of viral subunits was examined extensively for F, G, M or N proteins. These antigens were shown to be important targets for cytotoxic T cells (WATHEN et al. 1989; ALWAN et al. 1993; TAYLOR et al.

1997; WHITEHEAD et al. 1999; GADDUM et al. 2003). The optimization of the T helper (Th) cell epitope of a RSV G protein even enhanced the IFNγ-mediated immune reactions in mice (PLOTNICKY-GILQUIN et al. 2002). Thus, the immune reactions to the vaccine were modulated towards the aspired CMI. Interestingly, subunit-based vaccines were able to induce partial local immunity in the upper respiratory tract despite parenteral inoculation (WATHEN et al. 1989; TAYLOR et al. 1997; PLOTNICKY-GILQUIN et al. 2000).
Studies investigating the immunogenicity of aMPV proteins were also conducted (GULATI et al. 2000; GULATI et al. 2001; SHARMA et al. 2004a; CHARY et al. 2005). Purified recombinant N and M proteins were applied intramuscularly or subcutaneously in combination with Freund´s incomplete adjuvant, or oculonasally with or without cholera-toxin B. Irrespective of route and adjuvant both proteins induced partial protection.

In all published studies large amounts of recombinant proteins had to be administered. They also had to be combined with carrier or adjuvant systems to induce protective immunity. The solely application of these subunit vaccines seems to be limited to experimental studies due to high costs of development, production and application.

2.2.2.2. Live vector vaccines

Live viral or bacterial vector vaccines are based on a replicating non- or low-pathogenic virus or bacterium. They encode the genes of interest, which are expressed following infection of the host organism. The genes of interest are cloned into the vector genome and code for immunogenic proteins of other pathogens. Live vector vaccines are capable of induction of protective immunity. Using the nasal or oral route of inoculation they may induce local mucosal CMI.

Examples for live vectors which were evaluated experimentally in poultry:

- turkey herpesvirus (BUBLOT et al. 2007),

- infectious laryngotracheitis virus (OKAMURA et al. 1994),
- fowl adenoviruses (CAVANAGH 2003),
- paramyxoviruses, e.g. Newcastle Disease virus (HUANG et al. 2003),
- Salmonella spp. (KONJUFCA et al. 2006) and
- fowlpox viruses (STEENSELS et al. 2007).

The latter is licensed for commercial use in poultry production, e.g. for vaccination against Newcastle Disease virus (BOYLE u. HEINE 1993).

One live viral vector vaccine was evaluated experimentally for protection of turkeys against aMPV infection. This vaccine system was based on a live fowlpox virus recombinant expressing the F protein of aMPV (QINGZHONG et al. 1994). Repeated inoculation of the vaccine by intramuscular or wing web route induced partial protection against challenge with virulent aMPV.

In the latter study the F protein was shown to be a potential candidate for genetically engineered vaccines. But the fowlpox vector displays the disadvantage of obligatory parenteral inoculation. This route of vaccine application is non-economical. It may not induce optimal mucosal immunity. Besides these disadvantages it is speculated that high incidence of already acquired immunity to fowlpox viruses or alternative live vectors in commercial poultry may hinder the effectiveness of this vector vaccine system (SHARMA 1999).

2.2.2.3. Live viral (vector) vaccines based on reverse genetics

Reverse genetics systems were initially designed for the directed manipulation of a viral genome by mutagenesis, replacement or deletion of single genes to explore the functions of specified viral proteins.

This technique was applied extensively to members of the family *Paramyxoviridae* to identify important common features of the molecular biology of the viruses (MARRIOTT u. EASTON 1999). More recent it was applied to aMPV-A (NAYLOR et

al. 2004) and aMPV-C (GOVINDARAJAN u. KAMAL 2006; QINGZHONG u. ESTEVEZ 2006). These studies revealed new insights into the function of viral proteins and the importance of integrity of distinct sequences. They also gave promising results towards the use of this technique for engineering of defined, stable attenuated aMPV vaccines. In the latter two studies glow fluorescence protein (GFP) was successfully inserted as a candidate gene to be expressed *in vitro* by the resembled, live and replicating virus particles.

Only few scientific work was done on the reverse genetics system for aMPV so far. But the system is considered to be a promising candidate for future vaccine development.

2.2.2.4. DNA-vaccines

The idea of DNA vaccination was funded on the observation that inoculation of naked DNA induced transient *in vivo* expression of the encoded proteins (WOLFF et al. 1990). This observation was confirmed by a large number of *in vitro* and *in vivo* expression studies (TANG et al. 1992; GERDTS u. METTENLEITER 2001). The most promising results were found following transfection of antigen presenting cells (APC). The APC expressed the foreign proteins and presented respective peptides via major histocompatibility complex (MHC) antigen presentation. This mechanism induces CMI as well as humoral immunity (TANG et al. 1992; DUNHAM 2002).

DNA vaccines are engineered by cloning of a specified protein-encoding sequence into a non-replicating eukaryotic expression (plasmid) vector. The sequences coding for immunogenic proteins or for single or combined epitopes of a pathogen are cloned. Modern molecular biological techniques allow the design of sequences that are optimized with respect to immunogenicity. The optimization may be achieved by directed design of host-accessible epitopes for recognition by defined subsets of immune cells or by optimization of host codon usage. Based on these techniques the efficacy and immunogenicity of DNA vaccines are continuously enhanced. The use of expression vectors also allows the transfection of more than one gene into cells.

Gene sequences coding for immunostimulating molecules, such as cytokines, and also CpG motifs may also be inserted into the vectors (RAMSAY et al. 1999; VANROMPAY et al. 2001; DALE et al. 2004; ROH et al. 2006). The short repetitive sequences of unmethylated CpG dinucleotides called CpG motifs are found ubiquitous in bacterial plasmid DNA. They are strong inducers of cell mediated host immune reactivity. Artificial inclusion of CpG motifs in DNA vaccines may inhance or modulate the overall immunogenicity of a DNA vaccine (CHEN 2000; JORGENSEN et al. 2003).

DNA vaccines display the following advantages:

- There is no need for aggressive adjuvants, such as mineral oil. Thus, no local adverse reactions at the site of application are provoked.
- The vaccine is not able to replicate or to revert to virulence. It is not introduced into the genome of the host or a virulent pathogen. It does not induce residual negative effects, sometimes observed following application of some live attenuated vaccines.
- DNA vectors have a long shelf life and are resistant to extreme chemical and physical influences. This is in contrast to recombinant protein vaccines.
- The development of new DNA vaccines can be easy and fastly achieved; mass production of DNA vaccines is uncomplicated and cost-effective.

In contrast to that the major disadvantages of DNA vaccines are the following:

- The sensitivity of DNA vaccines to ubiquitous nucleases hampers the application to mucosal sites.
- This sensitivity to nucleases strongly increases the needed amount of DNA to be applied. Up to 90 % of applied DNA is destructed before uptake by potential expressing cell types, irrespective of the chosen route of application (BABIUK et al. 2000).

DNA vaccines have been tested experimentally in a variety of host organisms (BABIUK et al. 2000; GERDTS u. METTENLEITER 2001). This vaccine system has

also been used for immunization against poultry pathogens (LOKE et al. 2005; HSIEH et al. 2007; KLOTZ et al. 2007; LE GALL-RECULE et al. 2007).

The F and the N protein-encoding sequences of aMPV were tested as DNA vaccines for their immunogenic and protective potential (TARPEY et al. 2001; KAPCZYNSKI u. SELLERS 2003). In these studies the F protein, but not the N protein of aMPV induced partial protection against aMPV-challenge following repeated intramuscular application. Thus, the F protein was found to be a potential candidate for a DNA vaccine based immunization of turkeys against aMPV infection.

2.3. Microparticles (MP) as a vaccine delivery system

2.3.1. MP are controlled release systems (CRS)

CRS are based on biodegradable polymers that serve as carriers for active molecules, such as hormones, antigens or drugs. By binding or inclusion the active molecules are combined with the CRS. Besides the controlled release the CRS is capable of protecting the respective active molecules. The CRS may be designed as biofilms, particles, granules, pills or implants. Following application the active molecules are released by the polymers in a time dependent manner. CRS are widely used in human pharmacology, e.g. for long-term substitution of hormones or for the application of a variety of drugs. They allow for easy application of sensitive molecules (LANGER 1990).

CRS can also be designed as microparticles (MP). MP are spherical particles with diameters up to 10 µm. They are designed to encapsulate a vaccine in an inner reservoir or to strongly bind vaccines to their surface or in their polymeric matrix.

Among all controlled release systems the MP are the most interesting carrier system for application of genetic engineered vaccines to commercial poultry, as they may be adopted for mass application via spray, drinking water or feed.

2.3.2. Components for MP production

The basic components of MP production are:

- the polymer that provides the MP matrix;
- the organic solvents used during production;
- surface active agents (surfactants);
- additives that influence the surface charge of the MP.

2.3.2.1. Polymers

Although a variety of polymers, such as chitosane, polyacrylate or polystyrene, were tested for the production of MP, poly (D,L-lactic-*co*-glycolic acid) (PLGA) was shown to be the most promising candidate for MP-production. Subsequently most work has been done on different chemical compositions of MP and PLGA mixtures. PLGA was confirmed to be biocompatible as it is totally cleared from the organism *in vivo* by ubiquitous enzymatic hydrolysis of the polymeric lactic-co-glycolic structures. The resulting lactides and glycolides are completely metabolized (BENDIX 1997; MIDDLETON u. TIPTON 2000). PLGA is extensively used commercially for medical purposes, such as production of implants and surgical suture material.

Based on the type or blend of PLGA the bound or encapsulated vaccine is released in multiple ways (CHALLACOMBE et al. 1992; IGARTUA et al. 1998; KANEKO et al. 2000; YUN et al. 2005; WANG et al. 2006). Solid MP release the vaccine by diffusion and also as a consequence of polymer degradation, namely hydrolysis. MP that encapsulate the vaccine in an inner reservoir, particularly those with relatively wide diameters but thin capsule, additionally may swell and crack due to osmotic effects.

The MP-preparation protocol may influence the characteristics of the produced MP. MP can be manufactured to initially release a high proportion of bound vaccine in a short time and subsequently display a long lasting slow release of the residual bound vaccine (LANGER 1990; CHALLACOMBE et al. 1992). The total time period of release can be regulated to last between days and months or even years (WOO et al. 2001; YUN et al. 2005).

2.3.2.2. Organic solvents

Organic solvents are used to dissolve the polymer during MP preparation. The most widely used solvents are ethyl acetate and dichloromethane (TINSLEY-BOWN et al. 2000). They effectively dissolve the PLGA, do not interfere with the surfactant solution nor leave toxic residues due to their low boiling point and strong

hydrophilicity. Most importantly they do not interfere with or degrade the genetically engineered vaccines. Although both solvents have a toxic potential they are accepted in pharmacy for the use in production of controlled release systems. They are readily and totally removed from finalized MP by washing and evaporation steps (WATTS et al. 1990; TINSLEY-BOWN et al. 2000). The evaporation of the organic solvents can be enforced by vacuum.

2.3.2.3. Surfactants

Surfactants are needed in the watery phase of MP production. During MP preparation they coat the outer polymer matrix. Thus, they prevent aggregation of the manufactured MP. Additionally they influence the structure and hydrophobicity of the MP surface and the accessibility to phagocytosis. The uptake of MP by phagocytic cells, such as macrophages, was shown *in vitro* to be enhanced by hydrophobic and rough surfaces (HOSHI et al. 1999; VILA et al. 2002).

2.3.2.4. Additives to influence the MP surface's charge

Additional chemicals may be dissolved in the watery surfactant phase during the preparation of MP that are plasmid DNA-loaded on their surface. The poly-cationic reagent cetyltrimethylammonium-bromide (CTAB) provides poly-cationic residues on the surface of the MP that efficiently bind DNA.

The binding efficacy of CTAB can be enhanced by the addition of further chemicals to the organic polymer phase. Polyethyleneimine (PEI) is the most common agent for this purpose. PEI neutralizes the excessive anionic charge of the polymer matrix (OSTER et al. 2005).

2.3.3. Techniques of MP production

There are two basic protocols for the preparation of PLGA-MP: the double-emulsion-technique and the spray-drying method.

Both protocols allow a selective MP-preparation with regard to a variety of parameters, such as size, loading, surface and release characteristics. These parameters are mainly influenced by the choice of PLGA-composition, type of organic solvent, type of surfactant, speed and intervals of homogenization. Also the concentrations of vaccine, polymer and surfactant in the respective solutions influence the results of MP-preparation (WATTS et al. 1990).

2.3.3.1. Double-emulsion-technique

The double-emulsion-technique is the most widely used technique in experimental studies dealing with PLGA-MP. The preparation protocol is readily established and there is no need for purchasing of expensive laboratory technology. The MP-preparation generally is based on two steps of homogenization and a final purification step (TINSLEY-BOWN et al. 2000). The homogenization is done with a conventional laboratory homogenizer, such as the Ultra-Turrax (IKA, Germany):

Step 1

The genetically engineered vaccine is dissolved in watery solution and the PLGA in an organic solvent. The watery solution is emulsified in the organic solution of PLGA. The homogenization splits the watery vaccine solution into droplets. These droplets are surrounded by the dissolved PLGA.

Step 2

The resulting vaccine-in-PLGA emulsion is then emulsified in the watery solution of the surfactant. This second homogenization results in a vaccine-in-PLGA-in-surfactant emulsion. The vaccine droplets are coated by dissolved PLGA. These PLGA-coated droplets are surrounded by the watery surfactant solution.

Step 3

Finally the double-emulsion is poured into a large volume of the watery solution of the surfactant. The toxic organic solvent passes from the resulting MP into the excessive watery solution of the surfactant due to its strong hydrophilicity. The

solution then is heated or exposed to vacuum to remove the organic solvent. The removal of the organic solvent will lead to a hardening of the PLGA-coating. Thus, a PLGA-capsule or PLGA-MP is formed around the vaccine molecules. The resulting MP are washed extensively in consecutive washing steps to remove excessive surfactant and further additives. Finally the MP are lyophilized for long-term storage.

The described protocol is used for encapsulation of vaccines (GEBREKIDAN et al. 2000). More recent protocols describe the procedure of binding DNA vaccines to cationic surfaces of PLGA-MP. This enhances the effectiveness of DNA vaccine-loaded PLGA-MP when compared to MP that encapsulate the DNA vaccine (OSTER et al. 2005). For the preparation of cationic MP poly-cationic reagents and also chemicals that neutralize the excessive anionic charge of the polymer's matrix are added to the first surfactant solution (see step 2 of the preparation protocol).

2.3.3.2. Spray-drying method

The spray-drying method is based on almost the same preparation protocol as the double-emulsion-technique. In contrast to the double-emulsion-technique the double-emulsion is sprayed into a vacuum during the last step of preparation. This leads to ready-to-use, lyophilized particles.

The toxic organic solvent is totally removed by the vacuum. Thus, the spray-drying greatly reduces the need for the final surfactant solution and also for laborious heating and washing steps. This method is widely used in pharmaceutical and biochemical industry applications. It is used for sensitive molecules which are highly susceptible to deterioration by heat, organic-solvents and mechanical impact (MU u. FENG 2001). This method has not been applied very often for experimental approaches studying MP-based genetically engineered vaccines, due to the high costs and high technical needs of this method (OSTER u. KISSEL 2005).

2.3.4. Principle of function of microparticle-based vaccines

Following application onto mucosal surfaces or via the intramuscular route, PLGA-MP are phagocyted by cells due to their particulate and foreign nature (JILEK et al. 2005). The stimulatory effect of MP on the phagocytic activity of macrophages can be regulated. The diameter of the PLGA-MP and the chemical and physical characteristics of the used PLGA may influence the phagocytic activity. In particular the hydrophobicity of the PLGA used and surface characteristics stimulate the phagocytic activity of macrophages (ELDRIDGE et al. 1991; HOSHI et al. 1999; DUVVURI et al. 2006). PLGA-MP with diameters up to 10 µm were shown to pass the mucosal barrier. They enter the lymphatic system and are taken up by phagocytic cells (ELDRIDGE et al. 1989).

Following phagocytosis the PLGA-MP release the encapsulated or bound molecules, such as plasmid DNA or recombinant proteins. Once plasmid DNA is released to the cytoplasm of the immune cell, the encoded proteins are expressed and further processed for MHC-mediated antigen presentation on the cells surface. This specific antigen presentation strongly induces CMI as well as humoral immunity (HEDLEY et al. 1998; KANEKO et al. 2000; LUZARDO-ALVAREZ et al. 2005). The antigen presentation by macrophages and other antigen presenting cells may strengthen the overall immune reaction (LUZARDO-ALVAREZ et al. 2005).

2.3.5. Why microparticles?

It was decided to apply genetically engineered vaccines in a new generation vaccination concept to turkeys. To overcome the known disadvantages of the sensitive plasmid DNA- and recombinant protein-vaccines, a protective carrier system had to be considered. PLGA-MP as carrier systems for genetically engineered vaccines have the following advantages:

- easy mass application via the oral and nasal route by spray, drinking water or feed

- low costs of production and storage; lyophilized vaccines may be stored at ambient temperatures with long shelf life

- directed application to mucosal surfaces with subsequent induction of protective local cellular and humoral immunity (KANEKO et al. 2000)

- stimulation of the uptake of the vaccine by phagocytic, antigen presenting cells (JILEK et al. 2005)

- MHC-mediated antigen presentation by phagocytic cells following uptake of loaded PLGA-MP

- adjuvant properties, which may enhance the immunostimulatory efficiency of encapsulated DNA vaccines as compared to vaccination with naked DNA vaccines (ELDRIDGE et al. 1991; IGARTUA et al. 1998; WANG et al. 2006)

- protection of bound or encapsulated vaccines *in vivo* against degradation by ubiquitous proteases or nucleases

- protection of encapsulated protein vaccines *in vivo* against neutralizing antibodies

- potential for multivalent vaccines with a mixture of different genetically engineered vaccines

- potential for prime-boost systems by a mixture of loaded PLGA-MP with different chronological release profiles (LANGER 1990; MORRIS et al. 1994; LUZARDO-ALVAREZ et al. 2005; WANG et al. 2006)

- potential for vaccination against multiple pathogens with a single application, based on a mixture of loaded PLGA-MP of different pathogen-specifity and different chronological release profiles

2.3.6. The use of microparticles in poultry

Only few studies on the use of PLGA-MP have been done in poultry. Porter et al. (1997) and Hoshi et al. (1999) examined the uptake of (polystyrene) latex-MP and PLGA-MP by the intestinal mucosa following oral administration. They demonstrated the transport of MP to the gut associated lymphoid tissue and the effective uptake of MP by phagocytic cells. In another study the distribution of particulate colloidal carbon and fluorescent PLGA-MP in the respiratory and intestinal tracts was examined following application to embryonated chicken eggs (JOCHEMSEN u. JEURISSEN 2002).

More recently PLGA-MP loaded with heterologous human IgG (HEDLUND u. HAU 2001) or formalin-inactivated *Salmonella enteritidis* (LIU et al. 2001) were tested in chickens. PLGA-MP encapsulating inactivated duck parvovirus (PALINKO-BIRO et al. 2001) were tested in ducks. These three studies revealed that a PLGA-MP-based administration of antigens or vaccines via the oral or the intramuscular route to poultry may induce specific immune reactions. It also provided partial protection against challenge with the respective pathogens.

Investigations on PLGA-MP as a carrier system for genetically engineered vaccines for immunizations in poultry have not been published yet. A study investigating a combination of PLGA-MP and genetically engineered vaccines is of interest, because both systems display desirable and complementary advantages. Additionally, both systems were independently shown to principally work in poultry.

3. Goals and objectives

The ultimative goal of the project presented here was the development and testing of a new generation vaccination strategy against aMPV infection in turkeys.

In order to reach this goal a challenge model for aMPV-A and -B infections with defined parameters had to be established. Studies on vaccination and challenge of turkeys with an attenuated vaccine strain and also virulent strains of aMPV-A and aMPV-B subtypes were conducted. The objectives of these studies were to understand the pathogenesis and the immune reactions to aMPV infection in turkeys. Parameters were selected that help to evaluate the local immune reactions in the URT – in particular cell-mediated immune reactions - to aMPV infections.

The establishment of the challenge model was a prerequisite to study in the second part of this project the newly developed vaccination strategy for safety, immunogenicity and protection against aMPV challenge infection.

The objective in the second part of the project was to design a prime-boost vaccination strategy based on a DNA vaccine that encodes for the aMPV fusion protein and on a homologous recombinant F protein vaccine. The design was conducted by the following steps:

- preparation, individual characterization and testing of the genetically engineered vaccines *in vitro* and *in vivo*;
- establishment of the PLGA-MP preparation protocols for the binding of the DNA vaccine to the MP surface and for the encapsulation of the recombinant F protein into MP;
- usage of the respective PLGA-MP as controlled release systems for the two genetically engineered vaccines.

The MP-vaccine combinations were evaluated by

- testing them *in vitro* for adverse effects and functionality;
- testing of the final vaccination strategy for adverse effects following oculonasal application to turkeys *in vivo*;
- testing for the induction of local and systemic immune reactions after application of the vaccination strategy to turkeys *in vivo*;
- testing of the protective power against virulent aMPV infection in the previously defined aMPV-challenge model after application of the vaccination strategy to turkeys *in vivo*.

4. Induction of local and systemic immune reactions following infection of turkeys with avian Metapneumovirus (aMPV) subtypes A and B

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Induction of local and systemic immune reactions following infection of turkeys with avian Metapneumovirus (aMPV) subtypes A and B

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Abstract

Most of the studies regarding the immunopathogenesis of avian Metapneumovirus (aMPV) have been done with subtype C of aMPV. Not much is known about the immunopathogenesis of aMPV subtypes A and B in turkeys. Specifically, local immune reactions have not been investigated yet. We conducted two experiments in commercial turkeys. We investigated local and systemic humoral and cell mediated immune reactions following infection with an attenuated vaccine strain of aMPV subtype B (Experiment I) and virulent strains of aMPV subtypes A and B (Experiment II). Turkeys infected with virulent aMPV strains developed mild respiratory signs while birds inoculated with the attenuated aMPV did not show any clinical signs. Virus neutralizing antibodies were detected locally in tracheal washes and systemically in serum as soon as 5–7 days post aMPV infection (PI) independent of the strain used. Virus neutralizing antibody titres peaked at 7 days PI and then antibody levels declined. The peak of serum ELISA antibody production varied between infected groups and ranged from 14 and 28 days PI. All aMPV strains induced an increase in the percentage of CD4+ T cell populations in spleen and Harderian gland at days 7 or 14 PI. Furthermore, as shown in Experiment I, infection with the attenuated aMPV-B strain stimulated spleen leukocytes to release significantly higher levels of interferons (IFNs), interleukin-6 and nitric oxide in *ex vivo* culture in comparison to virus-free controls up to 7 days PI ($P < 0.05$). As detected by quantitative real time RT-PCR in Experiment II, infection with virulent aMPV induced an increased IFNγ expression in the Harderian gland in comparison to virus-free controls. IFNγ expression in the spleen varied between aMPV strains and days PI. Overall, our study demonstrates that aMPV subtypes A and B infection induced humoral and cell mediated immune reactions comparable to subtype C infections. We observed only temporary stimulation of serum virus neutralizing antibodies and of most of the local immune reactions independent of the aMPV strain used. The temporary character of immune reactions may explain the short duration of protection against challenge following aMPV vaccination in the field.

Keywords: Avian Metapneumovirus; Immunopathogenesis; Turkeys; Harderian gland

Abbreviations: aMPV, avian Metapneumovirus; BSA, bovine serum albumen; CD, ciliostatic dose; CEF, chicken embryo fibroblasts; CMI, cell mediated immunity; CT, cycle threshold; CTL, cytotoxic T lymphocyte; FBS, fetal bovine serum; HG, Harderian gland; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; NK, natural killer (cells); NO, nitric oxide; PBS, phosphate-buffered saline; PI, post inoculation; RT-PCR, reverse transcriptase polymerase chain reaction; QRT-PCR, quantitative real time RT-PCR; (S/P)-ratio, sample to positive ratio; TCID, tissue culture infectious dose; Th, T helper (cells); TOC, tracheal organ culture; VN, virus neutralization; VSV, vesicular stomatitis virus

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1. Introduction

Avian Metapneumovirus (aMPV) infection of turkeys, also known as avian or Turkey Rhinotracheitis, primarily affects the upper respiratory tract. Infected birds display coughing, clear to turbid nasal discharge, foamy conjunctival secretions and infra-orbital swelling (Cook et al., 1993; Van de Zande et al., 1999). The virus may cause immunosuppression, and thus field infections are often exacerbated by secondary bacterial infections (Van de Zande et al., 2001; Jirjis et al., 2004). This results in condemnations at slaughter and thereby in serious economic losses, as the morbidity is high in all infected age groups. Protection against aMPV infection in the field can be achieved by vaccination with live attenuated and also inactivated vaccines (Naylor et al., 2002; Patnayak et al., 2002; Van de Zande et al., 2002). aMPV has a non-segmented, negative-sense, single-stranded RNA genome and belongs to the family *Paramyxoviridae*, subfamily *Pneumovirinae* (Pringle, 1998). The virus was first described in South Africa in 1978 (Buys and Du Preez, 1980), and since then it has spread to a wide range of countries, as reviewed by Cook (2000). Subtypes are classified based on the nucleic acid sequence coding for the glycoprotein (Juhasz and Easton, 1994; Toquin et al., 2000). In Europe predominantly subtypes A and B, in particular cases subtype D, and in the USA subtype C are found.

Most work on aMPV immunopathogenesis has been done with aMPV subtype C. Previous observations in the field and in experimental studies indicate that aMPV displays an immunosuppressive effect (Timms et al., 1986; Jones et al., 1992; Chary et al., 2002c). aMPV subtype C may mediate inhibition of T cell responsiveness in the acute phase of infection (Chary et al., 2002b). Furthermore, infection with aMPV-C was shown to stimulate the accumulation of local CD4+ T cell subpopulations (Sharma and Gerbyshak-Szudy, 2002) and the release of CMI related cytokines (Chary et al., 2002a; Sharma et al., 2004). Almost no work has been done on the immunopathogenesis and the role of CMI in aMPV-A and -B infection of turkeys.

This study was designed to give new insights into the immunopathogenesis of aMPV-A and -B infection in turkeys. The emphasis of this study was placed on the comparison of local respiratory versus systemic immune reactions following infection with virulent aMPV-A and -B and attenuated aMPV-B. The following parameters were investigated: percentage of T-cell subpopulations in Harderian gland (HG) and spleen; production of bioactive cytokines and levels of intracellular cytokine transcripts; local and systemic aMPV-specific antibody production; detection of aMPV in the upper respiratory tract during the course of infection.

2. Materials and methods

2.1. Turkeys

One-day-old female commercial BUT white turkey poults were reared in positive-pressurized animal units following the guidelines of the Animal Care Committee of the University of Veterinary Medicine Hannover. Each experimental group was housed in separate animal units. Water and food were provided *ad libitum*.

2.2. Viruses

A lyophilized attenuated commercial vaccine (subtype B, strain VCO3) was reconstituted as recommended by the manufacturer. Each bird was inoculated with 10^4 tissue culture infectious doses $(TCID)_{50}$ per bird oculonasally (Experiment I). The virulent aMPV subtype A strain BUT 8544 and virulent Italian subtype B strain (kindly provided by Dr. R.C. Jones, Liverpool, UK) were propagated and titrated in chicken tracheal organ culture (TOC; Cook et al., 1976). Birds received 10^3 ciliostatic doses $(CD)_{50}$ per bird oculonasally (Experiment II).

An aMPV subtype A strain BUT 8544 (Wilding et al., 1986), which was adapted to chicken embryo fibroblasts (CEF), was propagated and titrated in CEF (Hafez, 1992) and used for virus neutralization (VN) test. Vesicular stomatitis virus (VSV; kindly provided by Dr. G. Zimmer, Institute for Virology, University of Veterinary Medicine Hannover, Germany) was propagated and titrated in CEF to be used in the IFN assay. Titres were determined by the method of Reed and Muench (1938).

2.3. Clinical score

According to Naylor et al. (1992), clinical signs were scored as followed: 0 = no clinical signs; 1 = clear nasal exsudate; 2 = turbid nasal exsudate; 3 = nasal exsudate with frothy eyes and/or swollen infra-orbital sinus.

2.4. H&E staining and patho-histological assessment

Harderian gland (HG), nasal turbinates and trachea were fixed in 10% phosphate-buffered formalin, and tissue sections were stained with haematoxylin and

eosin (H&E). Microscopically observed lesions were categorized as negative (0) or positive (1) based on lesions such as conspicuous interstitial heterophilic and lymphocytic infiltration and aggregation, destruction of epithelial and glandular tissue, and accumulation of detritus and exsudates in the lumen (Jones et al., 1986; Majo et al., 1996).

2.5. Tracheal washings

The entire trachea was clamped, removed and washed five times with idem volume of 1 ml phosphate-buffered saline (PBS, pH 7.2) containing 100 U penicillin/ml and 100 μg streptomycin/ml, using a 1 ml syringe and a 0.7× 30 mm injection needle (Suresh and Arp, 1995; Khehra, 1998). The trachea was massaged gently for increased washing effect. Following centrifugation, the supernatant was stored at −20 °C until further use.

2.6. ELISA and virus neutralization (VN) test

Sera were tested for aMPV specific antibodies by using a commercial aMPV ELISA kit (ART Ab Test Kit©, BioCheck B.V., Gouda, Holland), detecting aMPV specific IgG antibodies. The manufacturer's directions were followed with the following modifications: sera were diluted 100-fold instead of 500-fold to increase the detection of low antibody levels. For detection of maternally derived antibodies sera were diluted 500-fold as recommended. ODs were obtained by measurement of absorbance at 405 nm with a microplate reader. Based on the ODs the sample to positive (S/P)-ratios were calculated and used to express the mean (S/P)-ratio per group and day post infection (PI).

Both sera and tracheal washings were tested for aMPV specific neutralizing antibodies in the VN test (Baxter-Jones et al., 1989; Obi et al., 1997; Cook and Cavanagh, 2002) using cell culture adapted aMPV subtype A strain BUT 8544 as the virus to be neutralized. Briefly, replicates of 50 μl of 2-fold serial diluted sera were incubated for 1 h at 37 °C with 50 μl medium containing 200 $TCID_{50}$ of aMPV and then transferred to overnight cultures of primary chicken embryo fibroblasts (CEF) in 96-well plates (Nunclon Δ©, Nunc, Wiesbaden, Germany). CEFs were monitored for cytopathic effects up to 7 days PI. Titres are expressed as log 2 of the reciprocal of the highest serum dilution resulting in 100% neutralization. The geometric mean titres (log 2) were determined per group and day.

2.7. aMPV detection in nasal turbinates by nested RT-PCR

Detection of aMPV in nasal turbinates was done by nested RT-PCR (Cavanagh et al., 1999). RNA was isolated from nasal turbinates using TRIzol© Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. RT was performed with the ImProm-II© RT system (Promega, Mannheim, Germany) according to the manufacturer's directions using random primers. First PCR was performed with primers G6- 5′-CTGACAAATTGGTCCTGATT-3′, G1+A 5′-GGGACAAGTATCTCTATG-3′ and G1+B 5′-GGGACAAGTATCCAGATG-3′. The nested PCR was performed with 2 μl amplicon of the first PCR and with primers G5- 5′-CAAAGAA/GCCAATAAGCCCA-3′, G8+A 5′-CACTCACTGTTAGCGTCATA-3′ and G9+B 5′-TAGTCCTCAAGCAAGTCCTC-3′ (Cavanagh et al., 1999). All primers were used at a final concentration of 200 nM. ExTAQ©-polymerase (TAKARA, Cambrex, Potsdam, Germany) was used for the nested PCR with the following thermal profile setup: 94 °C for 5 min and 30 cycles of 94 °C for 1 min, 54 °C for 45 s and 72 °C for 45 s.

Following agarose gel electrophoresis the gel was stained with ethidium bromide and bands were visualized with an ultraviolet transilluminator.

2.8. Isolation and ex vivo stimulation of leukocytes

Leukocytes were isolated by density centrifugation (Archambault et al., 1976) of single cell suspensions. Briefly, pools of HGs from two birds and individual spleens were disrupted using a 70 μm Nylon cell strainer (BD Falcon©, Heidelberg, Germany). Leukocytes were collected from the interphase after density centrifugation on Biocoll© (1.09 g/ml; Biochrom AG, Berlin, Germany), washed and resuspended in medium given below.

For *ex vivo* cultivation, 2×10^6 cells/cm^2 of culture plate were incubated in 6- or 24-well plates (Nunclon Δ©, Nunc) in a humidified atmosphere at 41 °C and 5% CO_2. Medium was composed of RPMI 1640 with L-glutamine, 200 U penicillin/ml, 200 μg streptomycin/ml and 10% fetal bovine serum (FBS, PAA, Pasching, Austria). Cells were either incubated with medium alone or stimulated with 5 μg lipopolysaccharide (LPS, Sigma, Taufkirchen, Germany)/ml medium (Rautenschlein and Sharma, 1999). After 48 h of incubation the supernatants were harvested and used for nitric oxide (NO) detection or stored at −20 °C for cytokine detection.

2.9. Flow cytometric analysis

For flow cytometric analysis cells were double-stained with mouse-anti-chicken-CD4 antibodies conjugated to phycoerythrin and mouse-anti-chicken-CD8α antibodies (Chan et al., 1988) conjugated to fluorescein (Southern Biotech, Biozol, Eching, Germany). The antibodies were diluted to a final concentration of 0.5 and 5 μg antibodies/ml, respectively, in PBS containing 1% bovine serum albumen (BSA). Briefly, 1×10^6 leukocytes isolated from spleen and pooled HGs were suspended in 30 μl of antibody solution, incubated for 30 min on ice, washed twice with PBS containing 1% BSA, fixed with paraformaldehyde solution (3% in PBS) and stored at 7 °C until analysis (Suresh et al., 1993). CD4+ and CD8+ specific staining of lymphocyte fractions was determined using a Beckman Coulter Epics XL©. Gates were designed to analyze overall live lymphocyte fraction in 5000 cells per sample based on forward and sideward scatter. Data are expressed as the median *x*-fold change in percentage of CD4+ and CD8+ cells of all gated lymphocytes in relation to the virus-free group.

2.10. Interferon (IFN) assay

The overall level of types I and II IFNs was determined using the vesicular stomatitis virus (VSV) protection assay (Vengris and Mare, 1973; Karaca et al., 1996). Briefly, overnight cultures of primary CEFs were washed and incubated in duplicates of 2-fold dilution series of supernatants of *ex vivo* cultivated leukocytes from Experiment I. After 24 h of incubation, supernatants were discarded and $10^{4.5}$ $TCID_{50}$ of VSV were added to each well. Following 2 days of incubation the protective titre was determined based on VSV-specific cytopathic effects. The protective titre of total IFNs is expressed as the reciprocal of the highest sample dilution resulting in 100% VSV protection.

2.11. Interleukin (IL)-6 assay

This assay was performed with the murine hybridoma cell line 7TD1 (Lynagh et al., 2000; kindly provided by Bernd Kaspers, Institute of Physiology, LMU München, Germany). RPMI 1640 supplemented with L-glutamine, 10% FBS, 100 U penicillin/ml and 100 μg streptomycin/ml was used as culture medium. For cell line propagation culture medium was additionally supplemented with human recombinant IL-6 (Sigma) to a final concentration of 1 ng IL-6/ml. To test for IL-6, 2-fold dilution series of supernatants of *ex vivo* cultivated spleen leukocytes were accomplished with the given IL-6 free medium. The diluted samples were plated in duplicates in 96-well plates. Also, duplicates of a 2-fold standard dilution series of human recombinant IL-6 were run with each experiment. The 1×10^5 7TD1 cells/100 μl were added to each well and cultured for 4 days in a humidified atmosphere at 37 °C and 4.2% CO_2. Following incubation, 50 μl 0.025 mM XTT sodium salt and 0.025 mM phenazine methosulphate solution (Sigma) were added to each well, and plates were incubated for additional 4 h. The plates were colorimetrically evaluated at 450 nm. Mean IL-6 concentrations were determined per day and group based on linear regression of IL-6 standard dilution series.

2.12. IFNγ ELISA

IFNγ was measured in supernatants of *ex vivo* cultured spleen leukocytes with a commercial chicken IFNγ ELISA test kit (Cytosets©, BioSource, Solingen, Germany) according to the manufacturer's directions, using Nunc-Immuno© F96-well plates (Nunc). ODs were obtained by measurement of absorbance at 450 nm with a microplate reader. Samples were diluted 8 to 32-fold to obtain ODs in the linear range of IFNγ standard-dilution series' regression curve. IFNγ concentrations were determined by regression analysis and expressed as *x*-fold change in mean IFNγ concentration per group and day in comparison to virus-free control group.

2.13. Nitric oxide (NO) test

Accumulation of NO in supernatants of LPS-stimulated spleen leukocytes was determined as described by Green et al. (1982). Briefly, supernatants were mixed 1:1 with Griess reagent (one part of 1% sulphanilamide in 2.5 % phosphoric acid mixed with one part of 0.1% naphtylenediamine dihydrochloride in deionized water). A sodium nitrite standard dilution series, beginning with a concentration of 500 nM, was run in duplicates with each experiment. Absorbance was measured at 570 nm with a microplate reader. By means of linear regression analysis the mean NO_2 concentration (μM) was calculated per group and day PI.

2.14. Quantitative real-time RT-PCR (QRT-PCR) to detect IFNγ expression

IFNγ transcript levels in leukocytes isolated from HG and spleen were quantified according to Kaiser et al. (2000). The NucleoSpin© RNA II Kit (Macherey-Nagel, Düren, Germany) was used to prepare total RNA from

1×10^7 isolated leukocytes, according to the manufacturer's protocol. IFNγ-specific forward primer 5′-TTCCTGATGGCGTGAAGAAGG-3′, reverse primer 5′-TCCACCAGCTTCTGTAAGATGC-3′ and probe 5′-6-FAM-ACAGCTCATTTGCAGCCTTGCGCT-TAMRA-3′ were designed to lie across intron–exon boundaries (Applied Biosystems, Foster City, CA, USA), based on the partial turkey IFNγ gene sequence (Accession Number AJ000725). The 28S rRNA-specific primer–probe set was designed according to Kaiser et al. (2000): forward primer 5′-GGCGAAGCCAGAGGAAACT-3′, reverse primer 5′-GACGACCGATTTGCACGTC-3′ and probe 5′-HEX-AGGACCGCTACGGACCTCCACCA-BHQ2-3′.

The Brilliant© one step QRT-PCR Master Mix Kit (Stratagene, Amsterdam, Holland) was used for RT-PCR according to the manufacturer's recommendations, resulting in a final probe and primer concentration of 200 nM each. 5 μl total RNA was used per 25 μl reaction, and RT-PCR was performed using the Stratagene's MX 4000 quantitative PCR system. The following thermal profile setup was used: 50 °C for 30 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 54 °C for 1 min.

The standard curve for IFNγ was based on dilution of plasmid pTuIFNγ (kindly provided by P. Kaiser, Institute for Animal Health, Compton, United Kingdom; Lawson et al., 2001) in the range of 10^{-5} to 10^{-8}. The 28S standard curve was based on total RNA isolated from turkey spleens and diluted in the range of 10^{-2} to 10^{-5}. Each sample was tested in duplicates. Non-template controls and $\log_{10}$ dilution series of standards were included in each run.

Quantitation was done as described by Kaiser et al. (2000). Briefly, the detected reporter fluorescence was normalized to the passive reference dye ROX and subsequently standardized with respect to the corresponding 28S rRNA level as an indicator for homology in RNA input. The data are presented as the median of corrected cycle threshold (CT) values subtracted from 40 – designated as: corrected (40 − CT) – or as the median mRNA fold change based on the calculated corrected (40 − CT). Medians were determined per group and day PI.

2.15. Experimental plan

2.15.1. Experiment I

Thirty-three-day-old turkeys were randomly distributed into two groups of 30 birds each and inoculated oculonasally with virus-free primary CEF culture supernatant (virus-free group) or vaccinated with 10^4 $TCID_{50}$ of VCO 3 per bird (group aMPV-B-vacc). All birds were monitored daily for clinical signs. On days 1, 3, 5, 7 and 14 post inoculation (PI) six birds per group were chosen randomly for necropsy, anaesthetized and exsanguinated by transection of the left *A. carotis* and *V. jugularis*. Tracheae, HGs and nasal turbinates were evaluated for histological lesions. Nasal turbinates were investigated for aMPV-genome by RT-PCR, and tracheal washings and sera were tested for aMPV antibodies. Leukocytes were isolated from HG and spleen. The percentage of CD4+ and CD8+ T cell populations in HG and spleen were determined by flow cytometric analysis. Furthermore, spleen cells were cultivated with or without LPS *ex vivo*. The resulting cell culture supernatants were used for NO or cytokine detection, respectively. At days of necropsy additional serum samples were taken from the remaining birds of all groups ($n \leq 10$).

2.15.2. Experiment II

Thirty-five-day-old turkeys were randomly distributed into three groups of 42 birds each and inoculated oculonasally with virus-free TOC supernatant (virus-free group) or inoculated with 10^3 CD_{50} of aMPV subtype A or B per bird (group aMPV-A or -B, respectively). All birds were monitored daily for clinical signs. On days 1, 3, 5, 7, 14, 21 and 28 PI six birds per group were chosen randomly for necropsy, anaesthetized and exsanguinated by transection of the left *A. carotis* and *V. jugularis*. Tracheae, HGs and nasal turbinates were evaluated for histological lesions. Nasal turbinates were investigated for aMPV-genome by RT-PCR, and tracheal washings and sera were tested for aMPV antibodies. Leukocytes were isolated from HG and spleen. The percentage of CD4+ and CD8+ T cell populations in HG and spleen were determined by flow cytometric analysis. QRT-PCR was performed with RNA isolated from leukocytes of spleen and HG to detect IFNγ- and 28S-transcripts. At days of necropsy additional serum samples were taken from the remaining birds of all groups ($n \leq 10$).

2.16. Statistical analysis

Differences between virus-inoculated and the corresponding virus-free groups were analyzed by Two-Sample *T*-Test and by Wilcoxon Rank Sum Test.

3. Results

3.1. Induction of clinical signs and lesions following inoculation with aMPV

In Experiment I, 1 out of 25 vaccinated birds showed clear nasal exsudate on day 4 PI (data not shown). In

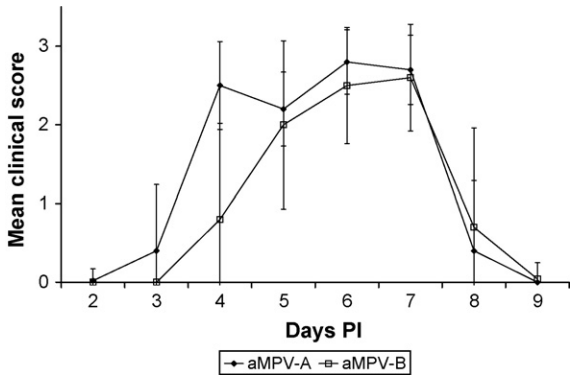

Fig. 1. Mean clinical score ± S.D. of aMPV infected turkeys per group on days post inoculation of 1×10^3 CD_{50} aMPV subtype A or B (Experiment II). Each bird was given a clinical score based on the monitored clinical signs: 0 = no clinical signs; 1 = clear nasal exsudate; 2 = turbid nasal exsudate; 3 = turbid nasal exsudate with frothy eyes and/or swollen infra-orbital sinus. The birds from the virus-free group showed no clinical signs at any time point (n = 10–53).

Experiment II, nasal exsudate, frothy eyes and/or swollen infra-orbital sinus were seen in 22% of aMPV-A infected birds on day 3 PI, and in 37% of aMPV-B infected birds on day 4 PI. On days 4 to 7 PI, 100% of aMPV-A infected birds showed clinical signs. Ninety-seven percent aMPV-B infected birds showed clinical signs on days 6 and 7 PI. At 10 days PI, none of the infected birds showed clinical signs anymore (data not shown). The mean clinical scores of both infected groups peaked between days 5 and 7 PI and dropped by day 9 PI as indicated in Fig. 1. None of the virus-free birds showed any clinical signs during Experiment I or II. At necropsy, no pathological lesions were seen in either experiment.

Inoculation of turkeys with vaccine and virulent strains of aMPV induced histological lesions in the upper respiratory tract, such as conspicuous interstitial heterophilic and lymphocytic infiltration and accumulation, destruction of epithelial and glandular tissue and accumulation of detritus and exsudates in the lumen of Harderian gland ducts, nasal turbinates or tracheae.

In Experiment I, 67 and 33% of the vaccinated birds showed microscopic lesions in the HG on days 3 and 7 PI, respectively. Lesions in the nasal turbinates were seen in 25 and 100% of the vaccinated birds on day 3 and on days 5 to 7 PI, respectively. No lesions were observed in the tracheae of vaccinated birds or in the upper respiratory tracts of virus-free birds (data not shown).

The development and incidence of lesions in the upper respiratory tract of virulent aMPV-A and -B infected birds is shown in Table 1. Lesions were found to be most severe in the mucosa of nasal turbinates. The physiological structure of the mucosa was nearly lost due to massive immune cell infiltration and aggregation. Mild heterophilic and lymphocytic infiltrates and minor damage of the epithelium with partial loss of ciliary integrity were found in the trachea. In HGs additional lesions, such as destruction of glandular tissue and accumulation of detritus in the lumen, were seen. After 7 days PI, the incidence of histological lesions decreased, and at 21 days PI no lesions were seen in the trachea anymore (Table 1).

3.2. Detection of aMPV-A or -B by nested RT-PCR

Up to 14 days PI viral RNA was detected by aMPV subtype specific nested RT-PCR in nasal turbinates of inoculated birds in both experiments (Table 2). No viral genome was detected in virus-free birds of either experiment.

Table 1
Patho-histological lesions in the upper respiratory tract of turkeys infected with virulent aMPV-A and -B (Experiment II)

Group	Organ	Percentage of birds with lesions/group[a] at days PI					
		3	5	7	14	21	28
aMPV-A	HG	50	75	100	17	33	0
	Nasal turbinates	100	100	100	83	66	17
	Trachea	25	100	100	50	0	0
aMPV-B	HG	17	83	83	50	50	20
	Nasal turbinates	100	100	100	50	50	20
	Trachea	0	100	87	33	0	0

[a] Birds were inoculated with 1×10^3 CD_{50} of aMPV subtype A or B. The microscopically observed lesions were assigned to negative or positive based on characteristic parameters such as interstitial heterophilic infiltration and lymphocytic aggregation, destruction of epithelial and glandular tissue and accumulation of exsudates in the lumen of the investigated tissue (n = 5–6). No lesions were seen in the upper respiratory tract of virus-free birds and of infected birds at day 1 PI.

Table 2
Detection of aMPV genome in nasal turbinates of aMPV infected turkeys by subtype-specific nested RT-PCR

Experiment	Group	Number of aMPV positive birds/total[a] at days PI				
		3	5	7	14	21
I	Virus-free	0/6	0/6	0/6	0/6	n.d.[b]
	aMPV-B-vacc	5/6	6/6	6/6	1/6	n.d.
II	Virus-free	n.d.	0/6	0/6	0/6	0/6
	aMPV-A	n.d.	6/6	6/6	5/6	0/6
	aMPV-B	n.d.	6/6	6/6	2/6	0/6

[a] Birds were inoculated with 1×10^4 $TCID_{50}$ of attenuated aMPV subtype B vaccine (Experiment I) or 1×10^3 CD_{50} of virulent aMPV subtype A or B (Experiment II).
[b] n.d.: not done.

3.3. Induction of local and systemic humoral immunity following inoculation with aMPV

In both experiments, all turkeys had detectable maternally derived antibody levels (mean $\log_{10}$ 3.69 ± 0.3) at 7 days of age. At 28 days of age 77 and 100% of the birds in Experiments I and II, respectively, had ELISA antibody titres below the recommended cut-off of S/P-ratio 0.2 and were considered ELISA antibody negative.

In Experiment I, all vaccinated birds developed detectable ELISA serum antibodies beginning at 5 days PI. The ELISA (S/P)-ratios increased up to 21 days PI (Table 3). The geometric mean VN antibody titre peaked at 7 days PI, followed by a decline up to 21 days PI (Table 4).

In Experiment II, the highest serum ELISA (S/P)-ratios of aMPV-A and -B infected birds were reached at days 21 and 28 PI, respectively (Table 3). Both infected groups reached their peak geometric mean titre of VN antibodies at day 7 PI, followed by a gradual decline up to day 28 PI (Table 4). No virus-free bird had detectable serum VN and ELISA antibodies after 5 days PI.

No antibodies were detected by the ELISA in tracheal washings in either experiment. Virus neutralizing antibodies were detected in tracheal washings from vaccinated and infected birds in both experiments. In tracheal washings of vaccinated birds VN antibodies were found up to 21 days PI, with the peak geometric mean titre (log 2) of 5.6 at 5 days PI (Experiment I; data not shown). After infection with virulent strains, aMPV antibody peak titres were reached between 7 and 14

Table 3
Serum ELISA antibody development after aMPV inoculation of turkeys

Experiment	Group	Mean S/P-ratio ± S.D.[a] per group at days PI				
		5	7	14	21	28
I	Virus-free	0.17 ± 0.09	0.15 ± 0.06	0.06 ± 0.07	0.08 ± 0.03	n.d.[b]
	aMPV-B-vacc	0.41 ± 0.27	0.41 ± 0.18*	0.93 ± 0.34*	1.21 ± 0.32*	n.d.
II	Virus-free	0.02 ± 0.06	0.03 ± 0.07	0.01 ± 0.04	0.02 ± 0.05	0.00 ± 0.02
	aMPV-A	0.04 ± 0.05	1.25 ± 0.28*	1.06 ± 0.47*	1.72 ± 0.67*	0.81 ± 0.40*
	aMPV-B	0.02 ± 0.05	1.53 ± 0.63*	3.32 ± 1.13*	3.19 ± 0.52*	3.99 ± 1.17*

[a] Birds were inoculated with 1×10^4 $TCID_{50}$ of attenuated aMPV subtype B vaccine (Experiment I) or 1×10^3 CD_{50} of aMPV subtype A or B (Experiment II).
[b] n.d.: not done.
* Significantly different to the virus-free group that day ($n = 6$; Two-Sample T-Test, $P < 0.05$).

Table 4
Induction of VN antibodies in serum of aMPV infected turkeys

Experiment	Group	Geometric mean titre (log 2) of VN antibody positive birds/group (% VN antibody positive birds/group)[a] at days PI				
		5	7	14	21	28
I	Virus-free	0 (0)	2.4 (37)	2.8 (17)	0 (0)	n.d.[b]
	aMPV-B-vacc	0 (0)	4.5* (100)	3.8* (100)	3.4* (86)	n.d.
II	Virus-free	2.2 (12)	0 (0)	0 (0)	0 (0)	0 (0)
	aMPV-A	3.5* (81)	9.2* (100)	6.8* (100)	6.6* (100)	6.4* (100)
	aMPV-B	3.5 (12)	5.9* (100)	5.4* (100)	4.0* (81)	3.1 (80)

[a] Birds were inoculated with 1×10^4 $TCID_{50}$ of attenuated aMPV subtype B vaccine (Experiment I) or 1×10^3 CD_{50} of aMPV subtype A or B (Experiment II). VN antibody titres ≥4 were considered positive.
[b] n.d.: not done.
* Significantly different to the virus-free group that day ($n = 6$–16; Two-Sample T-test, $P < 0.05$).

Table 5
Induction of VN antibodies in tracheal washings of aMPV infected turkeys (Experiment II)

Group	Geometric mean titre (log 2) of VN antibody positive birds/group (% VN antibody positive birds/group)[a] at days PI				
	5	7	14	21	28
Virus-free	1.7[b] (17)	0 (0)	3.0[b] (17)	0 (0)	0 (0)
aMPV-A	4.7[*] (100)	8.7[*] (100)	7.8[*] (100)	7.3[*] (100)	7.0[*] (100)
aMPV-B	5.3[*] (100)	7.7[*] (100)	7.8[*] (100)	6.9[*](100)	6.4[*] (100)

[a] Birds were inoculated with 1×10^3 CD_{50} of aMPV subtype A or B.
[b] Low "positive" titres may be due to non-specific virus neutralizing effects in supernatant of tracheal washings.
[*] Significantly different to virus-free group on that day ($n = 6$; Two-Sample *T*-Test, $P < 0.05$).

days PI, respectively, followed by a gradual decline (Table 5).

3.4. Stimulation of local and systemic cell mediated immune reactions following inoculation with aMPV

Virulent and vaccine aMPV strains induced an increase of local CD4+ T cells in the HG of inoculated birds at days 7 and 14 PI, respectively (Table 6). The percentage of CD4+ T cells in spleen was significantly increased after aMPV-vaccination at 14 days PI ($P < 0.05$) and was slightly increased in spleens of aMPV-infected birds at 7 and 14 days PI. No differences were observed in the percentage of local and systemic CD8+ T cell populations of aMPV-inoculated birds in comparison to virus-free controls in either experiment (data not shown).

3.5. Detection of total IFNs, IFNγ and IL-6 expression and release of NO following inoculation with aMPV

Inoculation of turkeys with attenuated aMPV-B (Experiment I) primed spleen cells to release IFNs, IL-6 and NO *ex vivo* (Pertile et al., 1995; Rautenschlein and Sharma, 1999). Spleen cells of aMPV-vaccinated birds, which were cultured *ex vivo*, released significantly increased levels of IFNγ and IL-6 at day 3 PI (Fig. 2b and c), in comparison to virus-free controls ($P < 0.05$). At 5 days PI, significantly increased levels of total IFNs and IL-6 were detected in spleen cell supernatants of aMPV-B vaccinated birds in comparison to cell cultures of virus-free birds (Fig. 2a and c; $P < 0.05$). Furthermore, *ex vivo* stimulation of spleen cells from infected birds with LPS induced a significant increase in NO production at 3 days PI in comparison to the virus-free group (Fig. 3; $P < 0.05$). At 7 days PI, all cytokine and NO levels in supernatants of *ex vivo* cultivated or LPS-stimulated spleen cells were comparable to the corresponding virus-free controls.

In Experiment II, the effect of aMPV infection on IFNγ transcript levels was investigated by QRT-PCR. Varying IFNγ mRNA levels were detected in spleen leukocytes of turkeys infected with virulent aMPV-A and -B, which were not significantly different to control birds (Fig. 4a; $P > 0.05$).

Locally in the HG infection with aMPV-A and -B induced up-regulation of IFNγ mRNA levels at days 5

Table 6
Effect of aMPV infection of turkeys on the percentage of CD4+ T cells in leukocyte preparations of HG and spleen

Organ	Experiment	Group	Median *x*-fold change of CD4+ T cells at days PI in comparison to virus-free birds[a]	
			Day 7	Day 14
HG	I	aMPV-B-vacc	1.0	4.3
	II	aMPV-A	2.1	0.7
		aMPV-B	3.6	0.3
Spleen	I	aMPV-B-vacc	1.0	1.7[*]
	II	aMPV-A	1.2	1.3
		aMPV-B	1.0	1.4

[a] Birds were inoculated with 1×10^4 $TCID_{50}$ of attenuated aMPV subtype B vaccine (Experiment I) or 1×10^3 CD_{50} aMPV subtype A or B (Experiment II).
[*] Significantly different to the virus-free group that day (HG: $n = 3$, no statistical test applicable; spleen: $n = 6$, Wilcoxon Rank Sum Test, $P < 0.05$).

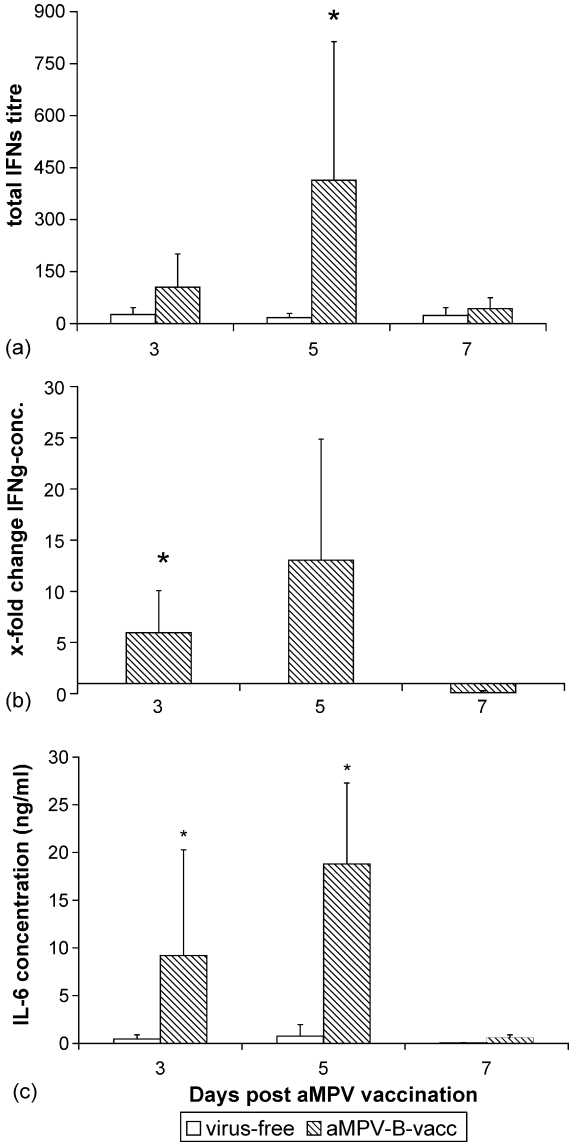

Fig. 2. Mean *ex vivo* release of total IFNs, IFNγ and IL-6 per day and group by spleen cells of turkeys inoculated with 1×10^4 $TCID_{50}$ of attenuated aMPV-B vaccine (Experiment I): (a) titres of total IFNs; (b) *x*-fold change of IFNγ concentration in comparison to virus-free controls; and (c) IL-6 concentration in supernatants from *ex vivo* cultivated spleen leukocytes. *Significantly different to the virus-free group ($n = 6$; Wilcoxon Rank Sum Test, $P < 0.05$).

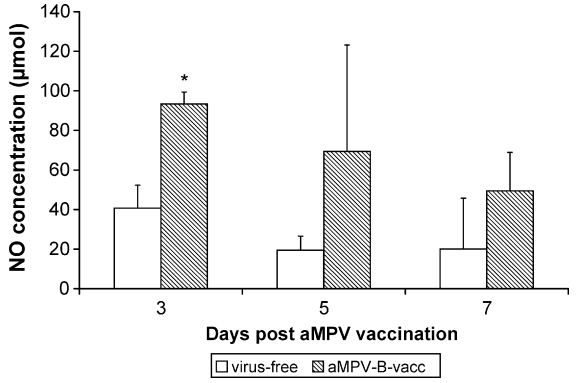

Fig. 3. Mean NO release per day and group of spleen cells of turkeys infected with 1×10^4 $TCID_{50}$ of attenuated aMPV-B vaccine (Experiment I). Nitric oxide was determined in supernatants of LPS-stimulated spleen leukocytes. *Significantly different to the virus-free group ($n = 6$; Wilcoxon Rank Sum Test, $P < 0.05$).

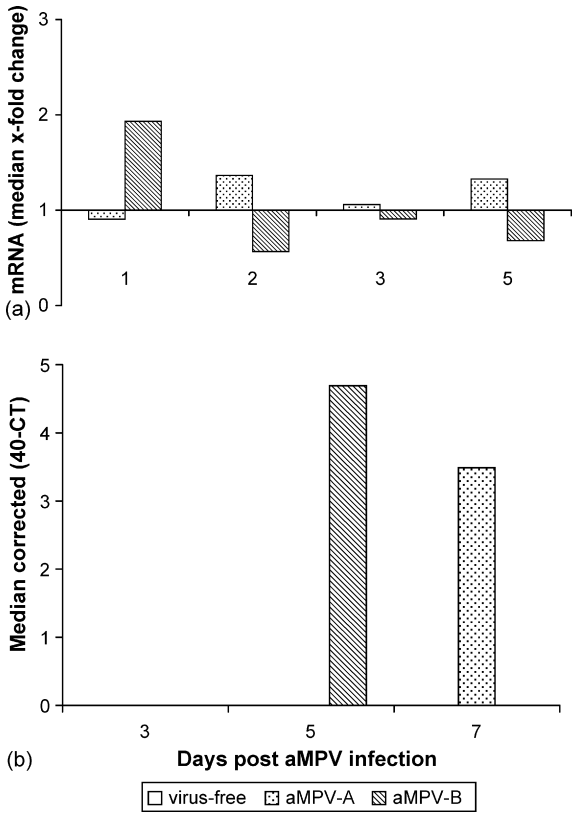

Fig. 4. Quantitation of IFNγ mRNA in (a) spleen leukocytes and (b) HG's leukocytes isolated from aMPV infected turkeys at days post oculonasal inoculation of 1×10^3 CD_{50} of aMPV subtype A or B (Experiment II; $n = 5$).

and 7 PI, respectively (Fig. 4b). No IFNγ mRNA was detected at any day PI in leukocytes isolated from HGs of virus-free birds.

4. Discussion

In the present study, we examined the immunopathogenesis of aMPV subtypes A and B in turkeys. In Experiment I birds were infected with an attenuated aMPV strain of subtype B. In Experiment II birds were infected with virulent aMPV strains of subtypes A and B. For the first time, parameters of local and systemic cell-mediated immune reactions were evaluated following

infection with subtypes A and B aMPV. We investigated the effect of aMPV-A and -B infection on local and systemic T-cell subpopulations, and the expression of CMI related cytokines. All aMPV strains induced a transient increase in the percentage of CD4+ T cells locally in the HG and systemically in the spleen, while the percentage of CD8+ T cell populations was not affected. Spleen leukocytes from birds infected with an attenuated aMPV subtype B strain released significant higher levels of bioactive, CMI related cytokines, such as IFNs and IL-6, and also NO, than leukocytes from virus-free birds up to 7 days PI ($P < 0.05$). Furthermore, infection with virulent aMPV strains induced an increase in IFNγ transcript levels in leukocytes isolated from HGs in comparison to virus-free controls. We also evaluated the local and systemic humoral immune response. The attenuated aMPV-B as well as the virulent aMPV strains induced an early onset of VN-antibody production in tracheal washes and serum at 5 to 7 days PI. The peak of serum ELISA antibody production varied between groups, and was detected between 14 days PI and 21 or 28 days PI in Experiments I and II, respectively.

The clinical outcome of infection with virulent aMPV strains was consistent with previous studies (McDougall and Cook, 1986; Cook et al., 1993; Naylor and Jones, 1994; Van de Zande et al., 1999; Marien et al., 2005). The phase of the severest clinical respiratory signs coincided with the peak of microscopical lesions. Furthermore, in this phase we observed an increase in the percentage of CD4+ T cells in HG and spleen, stimulation of IFNγ expression in the HG, and the detection of aMPV genome in 100% of the infected birds. The detected IFNγ transcript levels in spleen leukocytes of aMPV-infected birds had no defined different pattern in comparison to virus-free controls. This result is different to observations made with aMPV-C infection (Sharma and Gerbyshak-Szudy, 2002; Sharma et al., 2004). Sharma and Gerbyshak-Szudy (2002) detected increased transcript levels of IFNγ by QRT-PCR not only in the HG but also in the spleen up to 7 days PI. Possibly differences in aMPV subtype, virulence of the virus and genetic background of the turkeys may influence the immune reactions significantly.

No severe clinical signs were observed in birds inoculated with attenuated aMPV-B. But still the peak of microscopical lesions coincided with the detection of aMPV genome. Interestingly, between days 3 and 5 PI, before the onset of microscopic lesions in infected birds, we observed the increased release of IFNs, IL-6 and NO by *ex vivo* cultured spleen leukocytes in comparison to virus-free birds. We demonstrated the increased *ex vivo* production of total IFNs in a VSV protection assay. This assay detects all types of IFNs. With an IFNγ-specific ELISA we confirmed significant up-regulation of IFNγ at 3 days PI. Interestingly, the detected IFNγ up-regulation did not completely coincide with the significant up-regulation of the antiviral activity in cell culture supernatants in the VSV protection assay at 5 days PI. Based on these results we conclude that also some type I IFN may have been detected by the VSV-protection assay. We did not have monoclonal antibodies against type I IFNs or IFNγ available to confirm this speculation. Type I IFNs are known to be produced by virus-affected cells to activate antiviral mechanisms and also to activate NK cells (Schultz et al., 2004). IFNγ was shown to be released by NK cells and Th1 cells, and is involved in activation of macrophages (Kaiser et al., 1998). Previous studies have shown that activated macrophages release IL-6 and NO (Suresh et al., 1995; Qureshi, 2003). Besides macrophages, also Th2 cells are known to produce IL-6 to induce B-cell activation (Schneider et al., 2001). We may speculate that all CMI-related cytokines examined in this study may influence the pathogenesis of aMPV infection significantly.

Our observations from both experiments suggest a correlation between the detection of aMPV-genome in the upper respiratory tract, stimulation of humoral immune reactions and the onset of lesions. In parallel to the first detection of aMPV-specific antibodies in serum and tracheal washings, lesions waned. With the onset of ELISA antibody production aMPV genome detection decreased and was negative after 14 days PI. We may speculate that the recovery of the respiratory mucosa will continue after the virus has been cleared (Cook et al., 1993; Majo et al., 1995).

The microscopic lesions in nasal turbinates and tracheae induced by aMPV-A and -B were comparable to those described before (Jones et al., 1986; Majo et al., 1995; Van de Zande et al., 1999; Marien et al., 2005). The aMPV-A and -B induced microscopic lesions in HGs were comparable to those observed in aMPV-C infection (Chary et al., 2002b). The lymphoid cell accumulation in the HG provides circumstantial evidence for the importance of the HG for local immune reactions following upper respiratory tract infections (del Cacho et al., 1992; Maslak and Reynolds, 1995). This local accumulation of immune cells was also observed after infections with other viral respiratory pathogens, such as infectious bronchitis virus (Montgomery et al., 1991). The reduction of aMPV-induced clinical signs correlated with the decrease in histological lesions and the beginning of

mucosal regeneration, which combines with a reduction in local CD4+ T cells after 7 days PI.

In our experiments, vaccine and virulent aMPV strains induced the production of local VN antibodies as soon as 5 days PI, and systemic antibodies as soon as 5 and 7 days PI, respectively. The titres reached their peak at 7 days PI followed by a subsequent slow decline. The induction of aMPV–ELISA antibodies was delayed as expected in comparison to the VN antibody production. At the time of detectable aMPV–ELISA antibody levels, clinical signs had already vanished. Considering the correlation between increasing ELISA titres and declining aMPV detection, we may speculate that the ELISA antibodies play a role in the aMPV clearance, as stated before by Khehra (1998). On the other hand, field and laboratory observations indicate that ELISA titres may not be sufficient for protection against field challenge (Jones et al., 1992). Despite high levels of maternal antibodies and vaccine-induced ELISA antibodies aMPV field infections may occur.

The antibody titres varied between the two subgroups in VN test and ELISA. This may be due to differences in the subtype-specificity of the applied assays. Hafez (1992) showed that cross-reactivity between subtypes is limited. In the VN test we used cell culture adapted aMPV-A as the virus to be neutralized by sera of aMPV-A or -B infected birds. We do not know what antigen was used by the manufacturer to coat the ELISA plates.

In conclusion, we demonstrated that the immunopathogenesis of aMPV subtypes A and B infection in turkeys is dominated by local immune reactions in the upper respiratory tract, but systemically we also detected stimulation of cytokine expression such as IFNs and IL-6. The induction of local and systemic humoral immunity, the development of lesions, and clearance of virus was comparable between virus strains of different virulence and subtype. We may speculate that differences in clinical disease between strains may be due to differences in the induction of cytokines systemically, as differences between our study and observations with subtype C aMPV indicate. These aspects have to be investigated further. This knowledge of the immunopathogenesis of aMPV and the differences between strains will be important in the future for the development of challenge models, which are lacking so far, and vaccine development.

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5. A genetically engineered prime-boost vaccination strategy for oculonasal delivery with poly ($_{D,L}$-lactic-co-glycolic acid) microparticles against infection of turkeys with avian Metapneumovirus

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A genetically engineered prime-boost vaccination strategy for oculonasal delivery with poly(D,L-lactic-*co*-glycolic acid) microparticles against infection of turkeys with avian Metapneumovirus

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Abstract

In this study we demonstrated the use of an oculonasally delivered poly(D,L-lactic-*co*-glycolic acid) microparticle (PLGA-MP)-based and genetically engineered vaccination strategy in the avian system. An avian Metapneumovirus (aMPV) fusion (F) protein-encoding plasmid vaccine and the corresponding recombinant protein vaccine were produced and bound to or encapsulated by PLGA-MP, respectively. The PLGA-MP as the controlled release system was shown *in vitro* to not induce any cytopathic effects and to efficiently deliver the F protein-based aMPV-vaccines to avian cells for further processing. Vaccination of turkeys was carried out by priming with an MP-bound F protein-encoding plasmid vaccine and a booster-vaccination with an MP-encapsulated recombinant F protein. Besides the prime-boost F-specific vaccinated birds, negative control birds inoculated with a mock-MP prime-boost regimen as well as non-vaccinated birds and live vaccinated positive control birds were included in the study. The MP-based immunization of turkeys via the oculonasal route induced systemic humoral immune reactions as well as local and systemic cellular immune reactions, and had no adverse effects on the upper respiratory tract. The F protein-specific prime-boost strategy induced partial protection. After challenge the F protein-specific MP-vaccinated birds showed less clinical signs and histopathological lesions than control birds of mock MP-vaccinated and non-vaccinated groups did. The vaccination improved viral clearance and induced accumulation of local and systemic CD4+ T cells when compared to the mock MP-vaccination. It also induced systemic aMPV-neutralizing antibodies. The comparison of mock- and F protein-specific MP-vaccinated birds to non-vaccinated control birds suggests that aMPV-specific effects as well as adjuvant effects mediated by MP may have contributed to the overall protective effect.

Keywords: Microparticles; Avian Metapneumovirus; Genetic engineered vaccines

1. Introduction

Avian Metapneumovirus (aMPV) infection of turkeys primarily affects the upper respiratory tract and causes turkey rhinotracheitis (TRT) [1–3]. aMPV may induce immunosuppression [4,5] possibly due to a transient inhibition of T cell responsiveness [6]. TRT is often exacerbated by secondary bacterial infections [7,8], which results in condemnations at slaughter and thereby in serious economic losses. Partial protection against aMPV infection in the field is achieved by vaccination with live attenuated and also inactivated vaccines [9–11]. Despite the given vaccination strategies, aMPV field infections may occur. Live attenuated vaccines are suspected to have some residual immunosuppressive effects [6,12] and there is a risk that they may revert to more virulent variants in the field [13]. Inactivated vaccines have the disadvantage needing to be administered parenterally, which does not efficiently induce the necessary respiratory immu-

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nity and is not suitable for vaccination of larger numbers of turkeys in commercial operations. It is suggested that cell-mediated immunity (CMI), particularly the local CMI, plays the major role in protection. Infection with aMPV subtype C was shown to stimulate the accumulation of local CD4+ T cell subpopulations in the Harderian gland [14], and the release of CMI-related cytokines [15]. Our studies indicate similar effects on local T cell populations and CMI-related cytokines by infection with aMPV subtypes A and B [16].

It is necessary to improve current vaccination strategies against aMPV. Future aMPV-vaccines need the following characteristics: induction of local immunity at the site of entry; no risk of residual immunosuppressive effects or reversion to virulence of the vaccine itself; and easy and save administration to large numbers of turkeys. This goal may be achieved by combining the safety of genetically engineered plasmid DNA vaccines and recombinant protein vaccines with a microparticle-based controlled release system. Microparticles (MP) are a suitable delivery system for local application, as they protect sensitive vaccines and additionally show adjuvant effects.

Preliminary studies have shown that the fusion (F) protein of aMPV, delivered by a recombinant fowlpox virus vector [17] or by a plasmid DNA vector [18,19], was immunogenic and induced some protection against challenge infection. But the fowlpox vector system essentially demands the non-economic parenteral route of inoculation, and its use in poultry production may be problematic due to high prevalence of immunity against the viral vector itself [20]. The plasmid DNA vector had to be inoculated parenterally, which may not be suitable for induction of local CMI at the site of virus entry.

In a prime-boost strategy in mammals the consecutive intramuscular application of plasmid DNA and microencapsulated homologous recombinant protein elicited a broad immune response [21]. The local delivery of plasmid DNA, protected by MP, allows in mammals local expression of an immunogenic protein and the induction of specific protective immunity at the mucosal site [22]. Additionally, plasmid DNA vectors may elicit active innate immunity due to CpG motifs [23]. Many studies in mammals have shown that the use of poly(D,L-lactic-*co*-glycolic acid) (PLGA) for the preparation of MP delivery systems for vaccines is save and effective [24,25]. PLGA-MP may bind the vaccine to its cationic surface [26] or may encapsulate the vaccine [27], which efficiently protect the vaccines from degradation *in vivo*. PLGA-MP can be designed to release the vaccines in a controlled and sustained manner [28], thus giving the possibility for inherent booster effects without repeated inoculations. Through their particulate manner and hydrophilic surface charge, MP serve as an intracellular delivery system. They mediate the uptake of the vaccine from the mucosa by phagocytic cells.

The safety and efficacy of an MP-based vaccination strategy has never been tested in turkeys, and even in mammals only limited information is provided about their use for local application via the respiratory route. The goal of our study was to develop a vaccination strategy against aMPV infection of turkeys that combines a genetically engineered prime-boost immunization protocol with a controlled release system for oculonasal application. For this purpose we constructed a plasmid DNA vaccine encoding for the immunogenic F protein of aMPV subtype A, and adsorbed it to cationic MP. For booster-vaccination we prepared a recombinant F protein that is homologous to the plasmid-encoded F protein, and encapsulated it into PLGA-MP. We tested our vaccination strategy for safety, induction of local and systemic immunity and protection in turkeys. The protective power of this immunization was tested by homologous challenge of immunized turkeys with virulent aMPV subtype A.

2. Materials and methods

2.1. Turkeys

One-day-old female commercial BUT white turkey poults were reared in positive-pressurized animal units following the animal welfare guidelines of the University of Veterinary Medicine Hannover. Specific pathogen-free (SPF) chickens (VALO®, Lohmann LSL-LITE) were purchased from Lohmann Tierzucht (Cuxhaven, Germany). Chickens were hatched and reared in positive pressure isolation units (Montaim Van Stratum, Kronsberg, Netherlands) for the duration of the study. Each experimental group was housed in a separate animal unit. Water and food was provided *ad libitum*.

2.2. Viruses

A lyophilized attenuated commercial vaccine (subtype B, strain VCO_3) was reconstituted and inoculated oculonasally as recommended by the manufacturer (Experiment I). The virulent aMPV subtype A strain BUT 8544 (kindly provided by Dr. R.C. Jones, Liverpool, UK) was propagated and titrated in chicken tracheal organ culture (TOC) [29]. For challenge infection birds received 10^3 ciliostatic doses $(CD)_{50}$ per bird oculonasally in Experiments I and II. For virus neutralization (VN) test aMPV subtype A strain BUT 8544 [30], which was adapted to chicken embryo fibroblasts (CEF), was propagated and titrated in CEF [31]. Titres were determined by the method of Reed and Muench [32].

2.3. Vaccine preparation

2.3.1. Cloning and production of plasmid DNA and recombinant protein

Total RNA was isolated from aMPV-A strain BUT 8544 infected CEF monolayer using TRIzol© Reagent (Invitrogen) according to the manufacturer's recommendations. The mRNA was transcribed to cDNA using the

SuperScript© II RT Kit and random primers (Invitrogen) according to the manufacturer's instructions. The following PCR setup for aMPV-A fusion (F) prepolypeptide specific amplification was designed based on the protocol by Jing et al. [33]. Reverse primer PCRF.1st 5′-AAATAACTTAACTGACATAAGCCAT-3′, according to nucleotides 1612–1636 at the 3′ end of the F protein nucleotide sequence, and a modified 5′ phosphorylated forward primer AF+ 5′-GGACAAGTAGGATGGATGTAAG-3′, according to nucleotides 2–23 at the 5′ end (accession number: Y14294), were used to allow unidirectional TA Cloning© into the eukaryotic expression vector pCR3.1©-Uni (Invitrogen). The ligation product was transformed into One Shot© competent cells (Invitrogen) and F protein positive clones were detected by the PCR given above. One clone was confirmed by sequencing and also by the successful expression of encoded F protein in CEF, and named pF. Large quantities of pF plasmid DNA were purified using NucleoBond© PC 10000 anion exchange columns (Macherey-Nagel) according to the manufacturer's recommendations. pF was eluted in RNAse- and DNAse-free water (pH 5.0) and stored at −20 °C until further use.

For production of recombinant F protein (recF) the F protein coding sequence from pF was subcloned into the prokaryotic expression vector pET-23d(+)© (Merck Biosciences). The sequence was amplified with forward primer 5′-GCCGGATCCGACGGGACAAGTAGGATGGATGTA-AG-3′, including underlined BamHI restriction site, and reverse primer 5′-CCGGCGGCCGCGTGTAACTT-AACTGACATAAGCCATGCT-3′, including underlined NotI restriction site, to allow direct cloning of double-digested products into pET-23d(+). Ligation products were transformed into competent BL21 (DE3) cells, and positive clones were detected by PCR with the latter primer pair given above. One clone was selected and F protein expression was induced with IPTG (isopropyl-β-D-thiogalactopyranosid) according to the plasmid-manufacturer's instructions. The expressed C-terminal His-Tag© sequence encoded by pET-23d(+) allowed the purification of appending soluble, native recF using Ni-NTA His-Bind© Resins (Novagen). Subsequently, the eluted recF was passed twice through NAP-25 columns (Pharmacia Biotech), eluted with double-distilled water and concentrated in a vacuum concentrator (Savant Instruments). The amount of recF was quantified using Bio-Rad Protein Assay (BioRad, Germany).

2.3.2. Preparation of poly(D,L-lactic-co-glycolic acid) microparticles

Plasmid DNA was adsorbed to cationic microparticles (MP) according to the methods of Oster et al. [26]. 300 mg of the PLGA Resomer© RG 504 S (Boehringer Ingelheim) were dissolved in 3 ml dichloromethane (DCM; Sigma), and 30 mg polyethyleneimine (PEI; Sigma) were dissolved in 2 ml of DCM. Both solutions were mixed; 0.5 ml phosphate-buffered saline (PBS) was added to the mixture and homogenized for 1 min at 13,000 rpm with an Ultra-Turrax© T8 Homogenizer (IKA, Germany). The emulsion was added to 25 ml of a 0.5% cetyltrimethylammonium-bromide (CTAB) solution in distilled water and homogenized for 1 min at 20,000 rpm. The double-emulsion was stirred for 12 h at room temperature to evaporate all residual organic solvent. The MP were washed four times with 5% sucrose (Sigma) solution in distilled water with repeated centrifugation for 20 min at 10,000 × *g*, and finally the pellet was lyophilized.

For adsorption of DNA to the MP, 100 mg of cationic MP were suspended in distilled water (pH 5.0) and 1 mg pF was added, resulting in a final concentration of 0.5 mg DNA/ml. Thus, the theoretical loading was 1% (w/w), given that all DNA is bound to the MP. Following incubation for 1 h on ice, the MP were washed twice with distilled water (pH 5.0), centrifuged at 8000 × *g* for 10 min and suspended in distilled water (pH 5.0). Due to occasional aggregation of the prepared MP, the suspension then was homogenized by ultrasonic treatment three times for 5 s on ice at medium amplitude and power, lyophilized, designated as MPpF and stored at +4 °C until further use. Also MP with adsorbed homologue control plasmid vector including a 1366 bp non-expressed sequence were prepared and designated as MPpM.

RecF was encapsulated in MP by the double-emulsion technique of Tinsley-Bown et al. [25]. Briefly, 150 mg of PLGA Resomer© RG 503 H (Boehringer Ingelheim) and 50 mg of PLGA Lactel© 50 DG 040 (Birmingham Polymers Inc.) were dissolved in 1.2 ml DCM. Following addition of 50 μg recF/120 μl, the mixture was homogenized with an Ultra-Turrax© T8 at 10,000 rpm four times for 15 s on ice with respective intermissions to allow chilling. The first emulsion was poured to a 7% (w/v) polyvinylalcohol (PVA; Sigma) solution in distilled water and once more homogenized as described for the first emulsion. The resulting double-emulsion was immediately poured into 50 ml of distilled water at 37 °C, and stirred for 1 h to evaporate all residual organic solvent. The MP, designated as MPrecF were washed three times with distilled water with repeated centrifugation for 10 min at 18,000 × *g*, the pellet was lyophilized and stored at +4 °C until further use. Also MP encapsulating similar amounts of the control protein bovine serum albumen (BSA; PAA, Austria) were prepared for mock MP-vaccination and designated as MP-BSA.

2.4. Vaccine characterization

2.4.1. Expression of pF and MPpF after transfection and phagocytosis

Confluent monolayers of CEFs were transfected with pF using TransFectin© Lipid Reagent (BioRad) as recommended by the manufacturer in order to demonstrate *in vitro* expression of F protein in avian cells. To demonstrate *in vitro* expression of encoded F protein following phagocytosis of MPpF by primary avian macrophages, chicken macrophages were isolated from primary leukocytes and cultivated *in vitro* as described elsewhere [34]. These macrophages were incubated in 24-well cell culture plates (Nunc Δ©, Nunc

A/S) with 250 μg of suspended MPpF per well for 3 h, washed twice with media and incubated for additionally 48 h in a humidified atmosphere at 41 °C and 5% CO_2. Following transfection or phagocytosis, expressed F protein was visualized using immunohistochemical staining procedures. Briefly, cells were fixed with paraformaldehyde (3%, w/v in PBS) and incubated with reconvalescent's sera of aMPV-infected turkeys, followed by incubation with goat-α-turkey-IgG/PO (Nordic Immunology, Netherlands) and diaminobenzidine (DAB) peroxidase substrate (DAB-Kit, Vector Laboratories, USA). Stained cells were examined by light microscopy.

2.4.2. Characterization of recF

Expression of recF was confirmed by SDS-PAGE and Western blot to PVDF membranes [35,36]. RecF was visualized on autoradiography film by binding of reconvalescent's sera of aMPV-infected chickens, followed by goat-α-chicken-IgG/PO (Nordic Immunology, Netherlands) and ECL Plus western blotting detection reagents (Amersham). CEF-adapted aMPV strain BUT 8544 was propagated in CEF monolayers and concentrated by precipitation with polyethyleneglycol 6000 (Merck, Germany) as described by Trepanier et al. [37]. This crude virus preparation served as positive control.

2.4.3. Characterization of MPpF and MPrecF

MPpF were characterized by calculation of size distribution of the MP and vaccine adsorption efficiency. Additionally the structural integrity and conformation (supercoiled, open circular and linear) of pF following each step of MPpF preparation, *in vitro*-release and assay of protection against degradation by incubation with DNAse was determined by gel electrophoretic analysis. For calculation of the adsorption efficiency of MPpF the quantity of non-bound DNA in the supernatant of the adsorption reaction was measured by UV photometry (GeneQuant pro, Biochrom) at 260 nm. Efficiency was indirectly determined by calculation of the percentage of non-bound DNA in supernatant in relation to the initial used amount of pF [26]. In an *in vitro*-study the stability of MP-bound pF during incubation in phosphate-buffered saline (PBS) at 37 °C and continuous agitation was determined as described by He et al. [28]. The stability of cationic MP-bound pF against DNAse I-induced degradation was evaluated by incubation of 5 mg MPpF with 15 U DNAse I (Sigma) in a total volume of 250 μl for 1 h and subsequent extraction of remaining pF in the MP-pellet, as described in detail elsewhere [26]. MPrecF were characterized by calculation of size distribution and total protein loading. The total protein loading of MPrecF was determined by hydrolysis of the MP in 0,2 M NaOH solution [38] and subsequent quantification of the amount of recF in the supernatant using Bio-Rad Protein Assay (BioRad, Germany). The total protein load was expressed as micrograms of recF encapsulated per milligram of MP. The particle size distribution of both preparations was analyzed using an Abakus particle counter (Klotz, Germany), and is expressed as the percentages of particles with given diameters of 1–10 and <1 μm.

2.4.4. Effect of pF on specific antibody production following parenteral inoculation

The ability of pF to induce humoral immune reactions was tested *in vivo* in SPF chickens, as no turkeys free of aMPV-specific antibodies or even SPF turkeys were available. Briefly, three 6-week-old SPF chickens were inoculated intramuscularly with pF. Four hundred micrograms of pF/100 μl H_2O (pH 5.0) were injected into the muscles of the plantar thigh three times with 1 week intervals. Blood samples were taken at each inoculation, and also 7 and 14 days following the last inoculation. Blood samples were also taken from three age-matched and non-treated control birds. Sera were tested for aMPV-specific virus neutralizing antibodies.

2.5. H&E staining and pathohistological assessment

HG, nasal turbinates and trachea were fixed in 10% phosphate-buffered formalin, and 4 μm-sections were stained with haematoxylin and eosin (H&E). Slides were examined for histopathologic lesions and for infiltration of immune cells, particularly for lymphocytic aggregations. Microscopically observed lesions were categorized as negative (0) or positive (1) based on lesions such as conspicuous interstitial heterophilic and lymphocytic infiltration and aggregation, destruction of epithelial and glandular tissue and accumulation of detritus and exsudates in the lumen [39,40].

2.6. ELISA and virus neutralization (VN) test

Sera were tested for aMPV-specific antibodies of IgG-type with a commercial aMPV ELISA kit (ART Ab Test Kit©, BioCheck B.V.), detecting aMPV-specific IgG antibodies. The manufacturer's directions were followed with the following modifications: sera were diluted 100-fold instead of 500-fold to increase the detection of low antibody levels. ODs were obtained by measurement of absorbance at 405 nm with a microplate reader. Based on the ODs the sample to positive (S/P)-ratios were calculated and used to express the mean (S/P)-ratio per group and day.

Sera were tested for aMPV-specific neutralizing antibodies in the VN test [41–43] using cell culture adapted aMPV subtype A strain BUT 8544 as the virus to be neutralized. Briefly, replicates of 50 μl of twofold serial diluted sera were incubated for 1 h at 37 °C with 50 μl medium containing 200 tissue culture infectious doses ($TCID_{50}$) of aMPV and then transferred to overnight cultures of primary CEFs. CEFs were monitored for cytopathic effects up to 7 days PI. Titres are expressed as $\log_2$ of the reciprocal of the highest serum dilution resulting in 100% neutralization. Titres ≥4 were defined to be positive. The geometric mean titres ($\log_2$) of VN antibody positive birds were determined per group and day.

2.7. aMPV detection in choanal swabs by nested RT-PCR

Birds were sampled with dry swabs at the choanal cleft. Swabs were stored in TRIzol© Reagent (Invitrogen) at −20 °C until further processing. Detection of aMPV in choanal swabs was done by aMPV subtype-specific nested RT-PCR [44]. RNA was isolated from choanal swabs using TRIzol© Reagent (Invitrogen) according to the manufacturer's recommendations. RT was performed with the ImProm-II© RT system (Promega) according to the manufacturer's directions using random primers. First PCR was performed with primers G6-5′-CTGACAAATTGGTCCTGATT-3′, G1 + A 5′-GGGACAAGTATCTCTATG-3′ and G1 + B 5′-GGGACAAGTATCCAGATG-3′. The nested PCR was performed with 2 μl amplicon of the first PCR and with primers G5-5′-CAAAGAA/GCCAATAAGCCCA-3′, G8 + A 5′-CACTCACTGTTAGCGTCATA-3′ and G9 + B 5′-TAGTCCTCAAGCAAGTCCTC-3′ [44]. All primers were used at a final concentration of 200 nM. TAQ DNA-polymerase (Peqlab, Germany) was used for the first and second nested PCR with the following thermal profile setup: 94 °C for 5 min and 30 cycles of 94 °C for 1 min, 54 °C for 45 s and 72 °C for 45 s.

Following agarose gel electrophoresis the gel was stained with ethidium bromide, and bands were visualized with an ultraviolet transilluminator.

2.8. Isolation and flow cytometric analysis of HG and spleen leukocytes

Leukocytes were isolated by density centrifugation [45] of single cell suspensions. Briefly, individual or pooled samples of HGs and spleens were disrupted using a 70 μm nylon cell strainer (BD Falcon©). Leukocytes were collected from the interphase after density centrifugation on Biocoll© (1.09 g/ml; Biochrom AG), washed and suspended in PBS containing 1% BSA.

For flow cytometric analysis the leukocytes were double-stained with mouse-α-chicken-CD4 (clone CT-4) antibodies conjugated to phycoerythrin and mouse-α-chicken-CD8α (clone 3–298) antibodies [46] conjugated to fluorescein (Southern Biotech), as previously described [47]. CD4+ and CD8+ specific staining of lymphocyte fractions was determined using a Beckman Coulter Epics XL©. Gates were designed to analyze overall live lymphocyte fraction in 5000 cells per sample based on forward and sideward scatter. Data are expressed as the mean percentage ± S.D. of CD4+ and CD8+ T cells per day and group.

2.9. Clinical score

During the entire experiment all birds were monitored daily for clinical signs. According to Naylor et al. [48] clinical signs were scored as followed: 0 = no clinical signs; 1 = clear nasal exsudate; 2 = turbid nasal exsudate; 3 = nasal exsudate with frothy eyes and/or swollen infra-orbital sinus. Based on the acquired scores, data are presented as the cumulative number of all clinical positive birds (clinical score ≥1) per total number of observations in the indicated observation period, or as the percentage of severely clinically positive birds (clinical score ≥2) per group and day post challenge.

2.10. Experimental plan

2.10.1. Experiment I

One-day-old turkeys were randomly divided into the following groups: non-vaccinated controls (C: $n = 24$), birds mock-vaccinated with MPpM (MP-MOCK: $n = 12$), birds vaccinated with F protein-encoding MPpF (FV: $n = 24$) and birds inoculated with the commercial attenuated live vaccine as positive controls (LV: $n = 24$). Birds of group MP-MOCK and FV were inoculated twice oculonasally with 2 mg of MPpM and MPpF, respectively, in 50 μl of distilled water (pH 5.0)/dose at the age of 3 and 11 days. At 18 days of age MP-inoculation was repeated with 4 mg of each MP preparation/100 μl. At the age of 26 days, the birds in group MP-MOCK and FV were boostered oculonasally with 5 mg of MP-BSA and MPrecF, respectively, in 200 μl of PBS per bird. Non-vaccinated control birds received virus-free TOC-supernatant synchronous to the MP-vaccinations. The live vaccinated group LV received one recommended dose of at least $10^{2.3}$ $TCID_{50}$ attenuated aMPV subtype A strain VCO_3 in 100 μl of PBS per bird oculonasally at 9 and 23 days of age. At 34 days of age, 12 birds of groups C, MP-MOCK, FV and LV were inoculated oculonasally with 10^3 CD_{50} of virulent aMPV subtype A per bird. All birds were monitored daily for clinical signs during the entire experiment. Birds were swabbed in the choanal cleft at all days of vaccine inoculation and in 2-day-intervals post challenge (PC). At 5 and 13 days PC, six birds per group were chosen randomly and exsanguinated for necropsy. Tracheae, HGs and nasal turbinates were evaluated for histological lesions. Choanal swabs were investigated for aMPV-genome by RT-PCR, and sera were tested for aMPV antibodies by ELISA and VN test. Leukocytes were isolated from HG and spleen, each in pools of two birds. The percentages of CD4+ and CD8+ T cell populations in HG and spleen were determined by flow cytometric analysis.

2.10.2. Experiment II

One-day-old turkeys were randomly divided into two groups: non-vaccinated controls (C: $n = 35$) and birds vaccinated with MPpF (FV: $n = 22$). Birds of group FV were inoculated oculonasally with 2 mg of MPpF in 50 μl of distilled water (pH 5.0)/dose at the age of 2 and 9 days, and with 4 mg MPpF/100 μl at 16 days of age. At the age of 23 days, birds in group FV were boostered oculonasally with 5 mg of MPrecF in 200 μl of PBS per bird. Non-vaccinated control birds received virus-free TOC-supernatant synchronous to the MP-vaccinations. At 31 days of age, 12 birds of group C and 10 birds of group FV were inoculated oculonasally with

10^3 CD_{50} of virulent aMPV subtype A per bird. All birds were monitored daily for clinical signs during the entire experiment. Birds were swabbed in the choanal cleft at the days of vaccine inoculation and in 2-day-intervals PC. At 7 days following the third MPpF inoculation (23 days of age), at 8 days following the MPrecF inoculation (day of aMPV-challenge), and 5 and 13 days PC, five to six birds per group were chosen randomly and exsanguinated for necropsy. Tracheae, HGs and nasal turbinates were evaluated for histological lesions. Choanal swabs were investigated for aMPV-genome by RT-PCR, and sera were tested for aMPV antibodies in ELISA and VN test. Leukocytes were isolated from HG and spleen of individual birds. The percentage of CD4+ and CD8+ T cell populations in HG and spleen were determined by flow cytometric analysis.

2.11. Statistics

Differences between groups were analyzed by two by two contingency tables ($P < 0.05$ and < 0.005) or by Wilcoxon rank sum test ($P < 0.05$).

3. Results and discussion

3.1. Characterization of the MP-based controlled release system for the aMPV-plasmid DNA vaccine

The cationic MPs were characterized with respect to the aspired use as the vaccine delivering system. Following preparation and lyophilization, cationic MP were analyzed for size distribution. Fifty-six percent of the MP had a diameter between 1 and 10 μm, and 42% had a diameter less than 1 μm. Thus, 98% of the MP had a particle size that could be phagocytosed and processed by local immune cells, as shown previously in mammals [49–51]. Following adsorption of pF to the cationic MP, an actual adsorption efficiency of 87% was calculated. Hence the corresponding estimated loading was 8.7 μg pF per 1 mg of cationic MP. Following each MP preparation step and also following *in vitro* incubation over time pF was isolated from the resulting MPpF and characterized by gel electrophoretic analysis. No adverse effects on the structural integrity of pF were seen. As demonstrated by He et al. [28] before, the supercoiled form of pF was the predominant structure of pF that was recovered from the treated MPpF (data not shown). MPpF were incubated *in vitro* with DNAse I to test for the protection of the plasmid DNA provided by the adsorption to the cationic MP. Subsequent gel electrophoretic analysis of the structural integrity of the remaining pF revealed that pF was efficiently protected by the cationic MP (data not shown), similar to the results of Oster et al. [26]. In conclusion, the overall characteristics of MPpF were shown to be comparable to MP preparations that were successfully used in previous studies [26,28]. We established a protocol for the production of MPpF that were optimal in particle size and that effectively protected the plasmid DNA adsorbed to the MP. We chose this protocol for the production of MPpF that were used in subsequent experiments.

3.2. In vitro expression of pF in avian cells following transfection with pF and phagocytosis of MPpF

Transfection was conducted to demonstrate the effective expression of the F protein in avian cells. Subsequently, avian macrophages were tested for *in vitro* phagocytosis of the MPpF and the consecutive effective expression of F protein following phagocytosis. These studies were conducted *in vitro* with chicken cells due to the lack of aMPV-antibody-free turkeys, of SPF turkeys or turkey cell lines.

After transfection of CEFs with pF, expression of the aMPV F protein was observed. Positive cells were found to form typical syncytia [52] within the CEF monolayer (Fig. 1A).

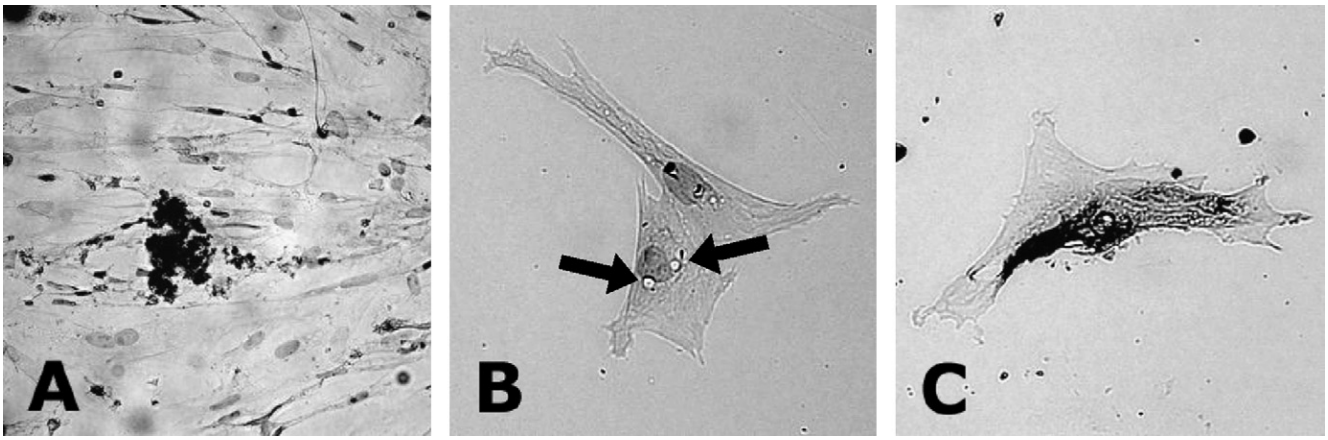

Fig. 1. Immunohistochemical detection of aMPV F protein expression by avian cells following transfection of CEFs with pF (A) or phagocytosis of MPpF (C) by primary chicken macrophages. (A) Syncytia-formation of CEFs following transfection with pF. (B) Unloaded MP are taken up by primary chicken macrophages; phagocytosed MP are indicated by arrows. (C) Primary chicken macrophages following phagocytosis of MPpF with subsequent expression of aMPV F protein (400×).

We demonstrated *in vitro* the phagocytosis of PLGA-MP by primary chicken macrophages (Fig. 1B) and also by chicken mononuclear cell line MQ-NCSU [53] (data not shown). Phagocytosed MP were seen in the majority of both types of macrophages as soon as 2 h after MP-administration. As demonstrated by Walter et al. [54] before, the phagocytosis of unloaded or loaded MP did not induce any cytopathic effect on the cells. The incubation with and the phagocytosis of MPpF by avian macrophages did not alter the cell's viability or death rate over time. During incubation of primary chicken macrophages with MPpF, MP were taken up and expressed F protein was detectable by aMPV-specific antibodies (Fig. 1C).

Therefore, we demonstrated that PLGA-MP were taken up *in vitro* by macrophages of avian origin and that MP-adsorbed plasmid DNA-encoded F protein was efficiently expressed. We speculate that MPpF may also be taken up *in vivo* from mucosal surfaces by respiratory phagocytic cells. The *in vivo*-uptake of polystyrene latex- or PLGA-MP into the intestinal lymphoid tissue of chickens or into the lympho-epithelium of conjunctiva-associated lymphoid tissue of turkeys has been shown before [55,56]. In mammals, the efficient *in vitro* uptake of loaded MP by dendritic cells or macrophages and subsequent expression of plasmid-encoded protein has been demonstrated before [54,57,58].

3.3. Immunogenicity of pF

To test the immunogenicity of pF *in vivo*, naked pF was inoculated intramuscularly to SPF chickens. A three-time-inoculation of naked pF with 1-week-intervals induced the production of aMPV-specific VN antibodies. At 0, 7 and 14 days following the last pF-inoculation two out of three inoculated birds had detectable aMPV-specific VN antibody levels (GMT of $\log_2$ 3.0 at the latter day) while control birds remained negative. Hence we confirmed that pF was immunogenic *in vivo*. In two previous studies, plasmid vaccines that encoded for the F protein of subtype C aMPV were used for intramuscular immunizations of turkeys [18,19]. In contrast to our study, in these previous studies the plasmid vaccines themselves did not induce detectable VN antibody levels. Instead, vaccinations had primed the birds to produce higher titres of antibodies following challenge with virulent aMPV than non-vaccinated did.

3.4. Characterization of the MP-based recombinant F protein vaccine

The recombinant F protein and the protein-encapsulating MP preparation were characterized *in vitro*. To test for the successful expression of the recombinant F protein, the protein was transferred after SDS-PAGE to a PVDF membrane by Western blotting. The blotted recombinant F protein was detected by an aMPV subtype A-specific chicken antiserum. It was specifically detected at molecular weights of approximately 70 and 50 kDa (Fig. 2). The 50 kDa band was the most prominent one detected by the aMPV-antiserum. The detected two protein bands of the PEG-concentrated aMPV subtype A had slightly lower molecular weight (Fig. 2). This might be explained by differences between the prokaryotic expression system for the recF and the expression of control virus in eukaryotic cells, particularly the formation of disulfide bonds and differences in glycosylation characteristics [59]. As first described by Cavanagh and Barrett [60], the aMPV Fusion protein gene is translated to a F_0 precursor protein of 68 kDa. F_0 is cleaved by cellular proteases to F_1 and F_2 subunits of a 53 and 15 kDa, respectively. These subunits form the activated disulfide-linked F_2/F_1 complex. Based on our results, we conclude that we successfully expressed the aMPV F_0 precursor protein, which was also cleaved into F_1 and F_2 subunits, as detected by the Western blot.

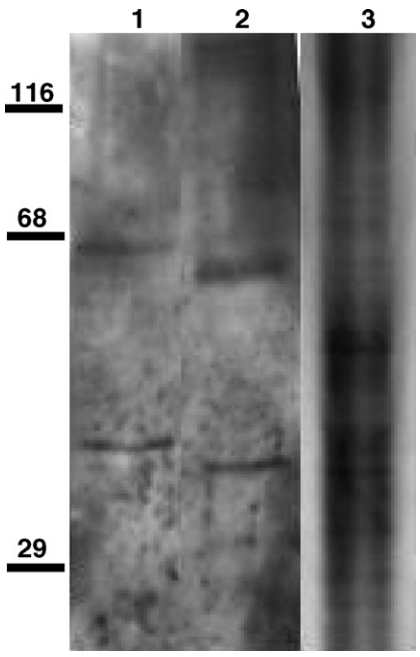

Fig. 2. Autoradiographic presentation of the Western blot analysis of the expressed recF and purified CEF-adapted BUT 8544. Bars indicate molecular weights (kDa); lane 1: recombinant Fusion protein (recF); lane 2: purified CEF-adapted strain BUT 8544; and lane 3: silver staining of purified CEF-adapted strain BUT 8544.

Following encapsulation of the recombinant F protein into MP, the size distribution and total protein loading of the resulting MPrecF were analyzed. Fifty-two percent of the measured MPrecF had a diameter between 1 and 10 μm, and 43% had a diameter less than 1 μm. Thus, 98% of the MP had the expected optimal size for phagocytosis [49–51]. MPrecF had a protein loading of 10 μg protein per milligram of MP, as determined by measuring the released protein in the supernatants of NaOH-hydrolyzed MPrecF. This protein load is low in comparison to previously published data of approximately 90 μg protein/mg MP [38,61]. The reason for that is not known. The recombinant F protein is low in solubility, and the high hydrophobicity may be disadvantageous for the encapsulation process. These characteristics of the recom-

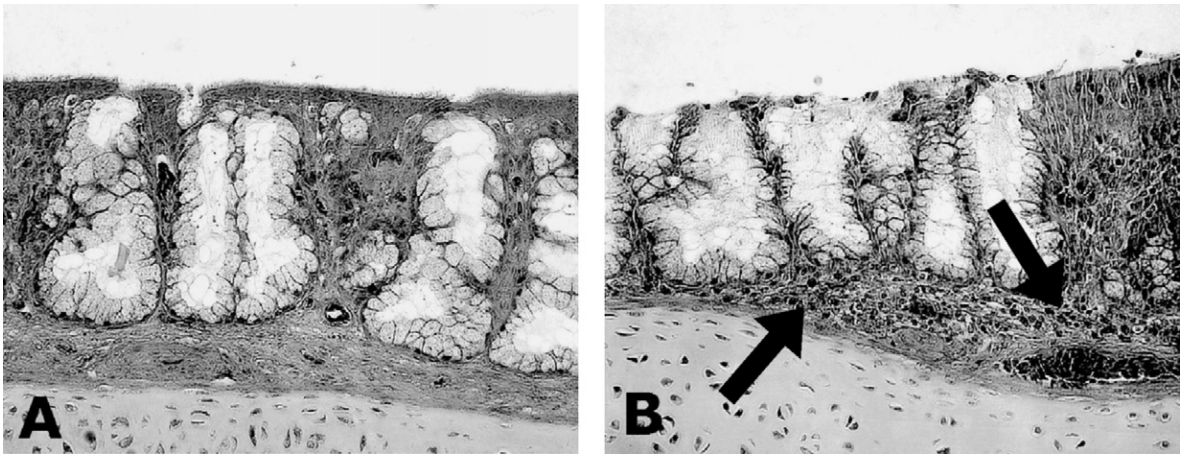

Fig. 3. Mucosa of non-vaccinated (A) and F protein-specific MP-vaccinated (B) birds' nasal turbinates at 8 days post MP-booster-vaccination (Experiment II). Note the infiltration of immune cells in F protein-specific MP-vaccinated birds' mucosa, as indicated by arrows (H&E, 200×).

binant F protein, particularly the formation of precipitates in highly concentrated solutions, also may give misleading results in the protein-assay.

3.5. Safety and immunogenicity of the prime-boost vaccination strategy with MPpF and MPrecF

We tested the MP-based plasmid DNA and recombinant F protein vaccines in a prime-boost strategy for safety and immunogenicity in turkeys. The safety and immunogenicity was evaluated in Experiments I and II exclusively before challenge and in non-challenged birds, respectively.

The oculonasal application of the MP-based vaccines did not induce any adverse effects, such as irritation of conjunctiva or nasal mucosa. No clinical signs, macroscopic lesions or histopathological lesions of HG, nasal turbinates or trachea were observed after MP-vaccinations in Experiments I and II. Immunization with the F protein-specific prime-boost system induced local cellular immune reactions. In Experiment II we observed conspicuous infiltration of immune cells histopathologically in HG of 33% and in nasal turbinates of 60% of the F protein-specific vaccinated birds at 8 days post MP-booster-vaccination (Fig. 3). This observation was in contrast to no infiltration in any examined tissue of the respective unvaccinated control birds.

Additionally, by flow cytometric analysis of HG's leukocytes in Experiment I we showed that at 13 days post MP-booster-vaccination 10.1% of the leukocytes from F protein-specific vaccinated birds were CD4+ T cells in comparison to only 4.2% in unvaccinated control birds. In Experiment II this observation was confirmed, although with 2.2% and 1.9%, respectively, the differences were less pronounced. The F protein-specific immunization also stimulated immune cells systemically, resulting in accumulation of CD4+ T cells in the spleen at 8 days post MP-booster-vaccination (Fig. 4). No cumulative effect on local and systemic CD8+ T cells was found in any vaccinated group (data not shown). Thus, the MP-based F protein-specific

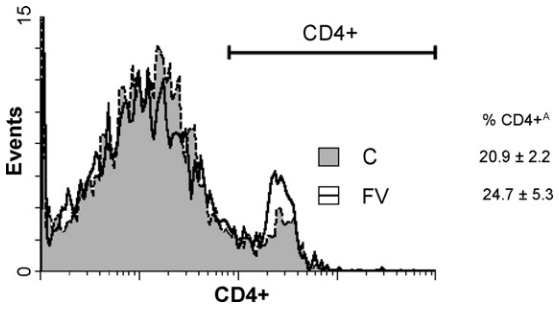

Fig. 4. Percentage of CD4+ T cells in spleens of F protein-specific MP-vaccinated (group FV) and non-vaccinated control birds (group C) in Experiment II. Representative example of CD4+ flow cytometric analysis of spleen leukocytes of birds from groups C and FV at day of challenge. [A]Mean percentage of CD4+ T cells per group and day ($n = 5$–6).

immunization induced local and systemic accumulation of CD4+ T cells.

During the vaccination period maternally derived antibodies had waned to a mean S/P-ratio of 0.2 ± 0.2 at 34 days of age in Experiments I and 0.9 ± 0.4 at 31 days of age in

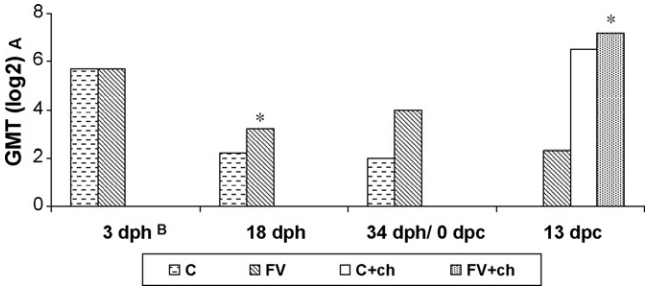

Fig. 5. Serum VN antibody development in birds of the F protein-specific MP-vaccinated group and of the non-vaccinated control group in Experiment I during vaccination (groups FV and C) and after aMPV challenge (groups FV + ch and C + ch). Birds were challenged with 1×10^3 CD_{50} of virulent aMPV. [A]The GMT (log_2) of serum VN antibody positive birds was determined per group and day. [B]dph: days post hatch; dpc: days post challenge. *Significantly different to the corresponding non-challenged or challenged, non-vaccinated control group ($n = 5$–10; Wilcoxon rank sum test, $P < 0.05$).

Experiment II. No aMPV-specific ELISA antibodies were induced by the prime-boost strategy. As shown in Fig. 5 the F protein-specific immunization reduced and even reverted the decrease of the serum VN antibodies in Experiment I. At the day of third MP inoculation (18 days of age) the GMT (log_2) of VN positive birds in F protein-specific immunized birds was significantly increased when compared to the non-vaccinated control group ($P < 0.05$). At 3 days of age all birds were VN antibody positive. At 18 days of age six out of six and three out of five and at 34 days of age (day of challenge) one out of six and one out of five birds of F protein-specific immunized and non-vaccinated control birds were VN antibody positive, respectively. The reason for the limited effect on VN antibody production and the lack of ELISA antibody production might be that the booster-immunizations were carried out in intervals that were too short for the development of humoral reactions or a booster effect. In mammals the booster-immunizations are carried out with time intervals of up to 3, 4 or even 16 weeks [22,28,62]. In our study the adjuvant effect of the vector and its CpG-motifs might have been reduced, because the intervals of 7–8 days between inoculations may have induced a refractory state of innate immune mechanisms [63]. Additionally, the total amount of plasmid DNA and recombinant F protein may have been too low to induce a systemic antibody response. The theoretically inoculated amount of 20 μg MP-bound pF per bird oculonasally in Experiments I and II was in contrast to 400 μg naked pF per bird inoculated intramuscularly in the preliminary *in vivo*-test of pF (see Section 3.3). Furthermore, the immunogenicity of recombinant F protein may have been low. Further improvement of the recombinant F protein may be needed. Optimization of codon usage or reduction of the aa-sequence to highly antigenic fragments of lower hydrophobicity, as demonstrated by Brown et al. [64], may optimize the booster effect.

3.6. Protective effects of the prime-boost vaccination strategy with MPpF and MPrecF against infection with virulent aMPV

We conducted two challenge experiments to test for the protective power of the MP-based genetically engineered prime-boost vaccination strategy against challenge with virulent aMPV.

All vaccinated groups showed significantly less clinical signs following challenge with virulent aMPV than non-vaccinated controls (Table 1). In Experiment I during the observation period of 3–9 days PC, the respiratory clinical signs were significantly reduced by 46% in F protein-specific vaccinated birds and by 100% in live vaccinated birds ($P < 0.005$), but were also reduced by 32% due to some adjuvant or non-specific effects in mock MP-vaccinated birds ($P < 0.05$), when compared to non-vaccinated control birds. There was no significant difference between mock MP-vaccinated and F protein-specific vaccinated birds ($P > 0.05$). The development of more severe respiratory clinical signs (clinical score ≥2) was delayed in F protein-specific vaccinated birds and these birds recovered earlier than control birds did in Experiment I (Fig. 6). At day 5 there was a significantly reduced number of clinical positive birds in F protein-specific vaccinated birds in comparison to control birds. This effect was confirmed in Experiment II (data not shown). No macroscopic lesions were found at necropsy in any group following challenge in Experiments I and II.

Histological lesions, such as conspicuous interstitial heterophilic and lymphocytic infiltration and accumulation, destruction of epithelial and glandular tissue and accumulation of detritus and exsudates in the lumen of HG's ducts, nasal turbinates or tracheae, were observed in all challenged birds of Experiments I and II. But in all vaccinated groups

Table 1
Respiratory signs of vaccinated and non-vaccinated turkeys[a] after challenge with virulent aMPV

Group	Cumulative number of clinically positive birds[b]/total observations in	
	Experiment I	Experiment II
C	37/60	58/95
MP-MOCK	25/60*	n.d.
FV	20/60**	25/50
LV	00/60**	n.d.

n.d.: not done. *$P < 0.05$ and **$P < 0.005$: significantly different to the challenged, non-vaccinated control-group (two by two contingency tables).

[a] Birds were inoculated with mock MP (group MP-MOCK), F protein-specific MP (group FV) and aMPV-live vaccine (group LV) or with virus-free TOC-supernatant (group C).

[b] Birds were challenged with 1×10^3 CD_{50} of virulent aMPV. Respiratory clinical signs were scored as followed: 0 = no clinical signs; 1 = clear nasal exsudate; 2 = turbid nasal exsudate; 3 = nasal exsudate with frothy eyes and/or swollen infra-orbital sinus; presented is the cumulative score of respiratory signs (clinical score ≥1) between days 3 and 9 PC.

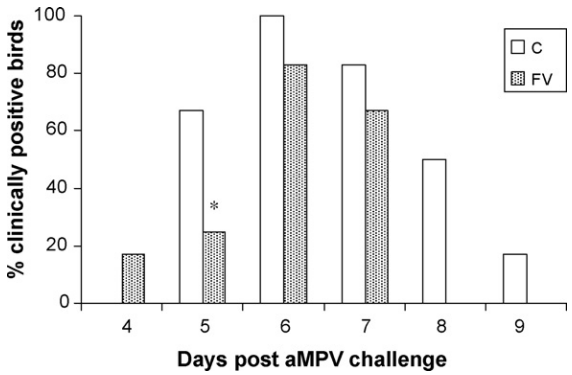

Fig. 6. Percentage of clinical positive turkeys with clinical score ≥2 in the F protein-specific MP-vaccinated group (group FV) and in the non-vaccinated control group (group C) in Experiment I after aMPV challenge. The respiratory clinical signs were observed each day post inoculation of 1×10^3 CD_{50} virulent aMPV. Each bird was given a clinical score based on the monitored clinical signs: 0 = no clinical signs; 1 = clear nasal exsudate; 2 = turbid nasal exsudate; 3 = turbid nasal exsudate with frothy eyes and/or swollen infra-orbital sinus. The birds from the non-aMPV-challenged groups and the aMPV-challenged, live vaccinated group showed no clinical signs at any time point ($n = 5$–17). *Significantly different to the challenged, non-vaccinated control-group that day (two by two contingency tables, $P < 0.05$).

Table 2
Pathohistological lesions in the upper respiratory tract of F protein-specific MP-vaccinated birds and non-vaccinated birds following challenge with virulent aMPV

Group	Percentage of birds with pathohistological lesions[a] in organs at days PC					
	HG		Trachea		Nasal turbinates	
	5	13/14[b]	5	13/14	5	13/14
C	83	27	33	8	100	73
FV	64	9	20	0	100	30

[a] Summary of Experiments I and II. Birds of the F protein-specific group (FV) and the non-vaccinated group (C) were challenged with 1×10^3 CD_{50} of virulent aMPV.
[b] Experiment I at 13 days PC and Experiment II at 14 days PC ($n = 10$–14).

the percentages of birds with observed lesions in the HGs, tracheae and nasal turbinates were reduced in comparison to the respective non-vaccinated control group. The reduction of histological lesions was most pronounced in birds immunized with the F protein-specific vaccination strategy (Table 2), whereas it was less prominent and less reproducible in mock MP-vaccinated and live vaccinated birds (data not shown). The F protein-specific vaccination apparently reduced the destruction of epithelium, glandular tissue and tissue architecture in HG, trachea and nasal turbinates due to aMPV challenge, when compared to challenged, non-vaccinated control birds.

Our observations indicate that the F protein-specific vaccination induced some local protection. The PLGA-MP may have some significant adjuvant-effects, because the mock MP-vaccination also induced some reduction of clinical signs and pathohistological lesions.

The effects of F protein-specific MP-vaccination on the accumulation of local CD4+ T cells in HG's leukocytes following challenge with virulent aMPV were comparable to the effects observed in non-challenged birds (as described in Section 3.5). In Experiment II in the F protein-specific vaccinated birds we found accumulation of systemic CD4+ T cells in spleens at 5 and 14 days following challenge, when compared to challenged, non-vaccinated birds (Fig. 7). No cumulative effect on local and systemic CD8+ T cells was found in any vaccinated group following challenge (data not shown).

Choanal swabs were examined for aMPV genome detection to test for an effect of the F protein-specific vaccination on the duration of viral shedding following challenge. Before challenge, none of the choanal swabs were found to be positive for aMPV subtype A or B, except for the swabs taken from live vaccinated birds. Those swabs were positive for the subtype B live vaccine at 9 and 16 days after the first and second live vaccination, respectively. Following challenge, in all vaccinated groups the numbers of birds that where positive for aMPV in choanal swabs were reduced, when compared to the non-vaccinated, challenged controls (Table 3). There was no statistical significant difference in the number of birds that were positive for aMPV in choanal

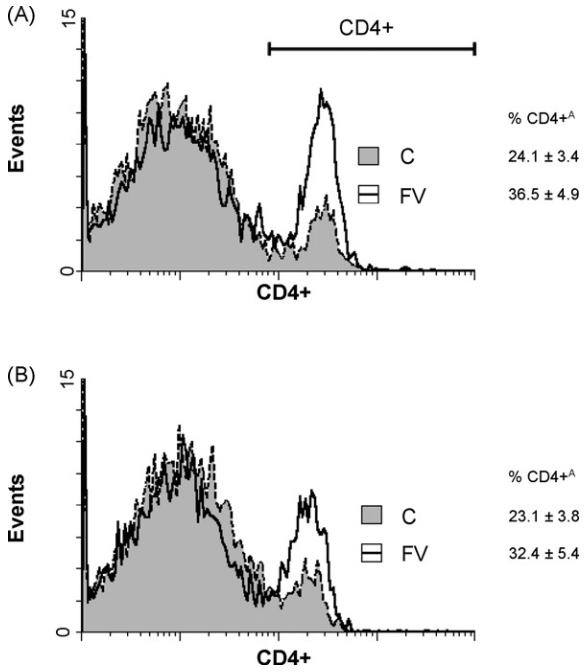

Fig. 7. Percentage of CD4+ T cells in spleens of F protein-specific MP-vaccinated (group FV) and non-vaccinated control birds (group C) following challenge with 1×10^3 CD_{50} virulent aMPV in Experiment II. Representative examples of CD4+ flow cytometric analysis of spleen leukocytes of birds from groups C and FV at 5 (A) and 14 (B) days PC. The percentages of CD4+ T cells in spleens of non-challenged, non-vaccinated control birds were 22.4 ± 2.8^A and 23.0 ± 2.6 at 5 and 14 days PC. [A]Mean percentage of CD4+ T cells per group and day ($n = 5$–6).

swabs of F protein-specific vaccinated birds at any day post challenge, when compared to those of mock MP vaccinated birds or to those of non-vaccinated, challenged control birds ($P > 0.05$). In F protein-specific vaccinated birds no aMPV genome was detected after 11 days PC and in live vaccinated birds after 5 days PC.

Table 3
Detection of aMPV genome by nested RT-PCR in choanal swabs from immunized turkeys[a] following challenge with virulent aMPV (summary of Experiments I and II)

Group	RT-PCR positives/total number of RNA-pools[b] at days post aMPV challenge		
	5 + 7	9 + 11	13/14[c]
C	16/18	10/18	2/9
MP-MOCK	9/9	2/6	1/3
FV	15/17	4/16	0/8
LV	1/9*	0/6*	0/3

[a] Birds were immunized with mock MP (group MP-MOCK), F protein-specific MP (group FV) and aMPV-live vaccine (group LV) or with virus-free TOC-supernatant (control group C).
[b] Birds were challenged with 1×10^3 CD_{50} of virulent aMPV. RNA was isolated from individual choanal swabs taken per bird and day. Individual RNA or RNAs pooled from two birds were used for RT-PCR and data were summarized for the indicated days.
[c] Experiment I at 13 days PC and Experiment II at 14 days PC.
* Significantly different to the challenged, non-vaccinated control-group that day (two by two contingency tables, $P < 0.05$).

Following challenge in Experiments I and II, no effects of the MP-vaccinations on systemic ELISA antibody production were observed, when compared to non-vaccinated groups (data not shown). The challenged, F protein-specific immunized birds showed significantly higher neutralizing aMPV antibody levels in comparison to challenged, non-vaccinated control birds at 13 days PC (Fig. 5). At 13 days post challenge two out of six non-challenged, F protein-specific immunized birds were VN antibody positive in contrast to no VN antibody positive bird in non-challenged, non-vaccinated control group (Fig. 5). At 13 days post aMPV challenge all birds in all challenged groups were VN antibody positive. The GMT ($\log_2$) of challenged, F protein-specific immunized birds was significantly higher than that of challenged, non-vaccinated control birds (Fig. 5).

The reduction of viral shedding in F protein-specific immunized birds in comparison to mock MP-vaccinated birds and non-vaccinated control birds may have been due to the increased VN antibody production or due to enhanced aMPV-specific CMI.

4. Conclusions

This is the first study in the avian system demonstrating the use of an oculonasally delivered, MP-based and genetically engineered vaccination strategy. aMPV-infection was used as a respiratory disease model. The PLGA-MP as the controlled release system were shown *in vitro* to efficiently deliver the F protein-based aMPV-vaccines to avian cells for further processing. The immunization of turkeys via the oculonasal route induced systemic humoral immune reactions and local and systemic cellular immune reactions. Following challenge with virulent aMPV we were able to demonstrate partial protection provided by the F protein-specific immunization. The F protein-specific vaccination reduced clinical signs and histopathological lesions, induced systemic VN antibody production, improved viral clearance and induced accumulative effects on local and systemic CD4+ T cells to a greater extend than the mock MP-vaccination did. In most cases the differences between the evaluated groups could not be statistically supported due to the limited number of birds evaluated per experimental day and individual variation between birds. The comparison of mock- and F protein-specific MP-vaccinated birds with the non-vaccinated control birds suggests that aMPV-specific effects, such as VN antibody production and enhanced aMPV-specific CMI, as well as adjuvant effects may have contributed to the overall protective effect. The supposed adjuvant-effects may be due to non-specific activation of mucosal and systemic immunity, as this effect has been shown before for PLGA-MP [26,61]. The CpG motifs in the plasmid DNA preparation, and the PLGA-MP themselves have substantial stimulatory effects [23,54,65]. These unspecific immune reactions are desirable protective effects in the period between vaccine inoculation and onset of protective specific immunity. As shown in mammals, DNA-loaded MP stimulate specific local and systemic humoral and cellular immune responses [22,28,66]. The involved immune reactions needed for protection against aMPV infection have to be explored in more detail in the future, particularly the contribution of vaccination to local, virus-specific T cell immunity. The promising results of this study encourage to further optimize this vaccination strategy, its schedule and genetically engineered components. This might allow for the *in vivo* application of this strategy for the protection against single or even multiple, economically important respiratory pathogens in poultry.

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6. Discussion and conclusions

The principle objective of the presented studies was to develop a new vaccination strategy against aMPV infection in turkeys. In order to evaluate the aspired vaccine candidates, the establishment of a defined challenge model for aMPV infection in turkeys was needed. Studies on the pathogenesis of aMPV were conducted. In these studies the pathogenesis of attenuated and virulent strains of aMPV subtypes A and B were compared. The parameters were chosen focusing on the assessment of local immune reactions in the URT and of CMI. Consecutively the evaluation of the developed new vaccination strategy was conducted with the challenge model based on these defined parameters.

6.1. Development and application of an aMPV-challenge model

Commercial turkeys were infected with attenuated aMPV-B, virulent aMPV-A or aMPV-B. Following infection clinical signs, macroscopical and microscopical lesions, detection of aMPV genome in tissues of the URT and humoral and cellular immune reactions were evaluated. Subsequently, the developed challenge model was applied to test the newly developed vaccination strategy by using a virulent challenge strain of aMPV subtype A.

6.1.1. Pathogenesis of aMPV-infection

The clinical signs observed during infection with either virulent aMPV strain tested were comparable to those described before (MCDOUGALL u. COOK 1986; COOK et al. 1993b; NAYLOR u. JONES 1994; VAN DE ZANDE et al. 1999; MARIEN et al. 2005). The microscopic lesions induced by aMPV-A and aMPV-B infections (JONES et al. 1986; MAJO et al. 1995; VAN DE ZANDE et al. 1999; MARIEN et al. 2005) and by aMPV-C infections (CHARY et al. 2002b) were extensively evaluated in previous studies. The observations on microscopical lesions in nasal turbinates, tracheae and HG following aMPV challenge were consistent with those studies. In contrast to aMPV-A and aMPV-B, infection with aMPV-C does not affect ciliar activity *in vitro*

(COOK et al. 1999). Whether this difference in modulation of ciliar activity and subsequently the reduced mucosal clearance does have an impact on differences in pathogenesis between aMPV subtypes *in vivo* is not known. It is speculated that the induction of clinical signs and local lesions is based on the same mechanisms for all three aMPV subtypes.

In the studies presented here aMPV genome was detected in nasal turbinates of infected turkeys by nested RT-PCR. At 14 days PI, 5 out of 6 in comparison to 2 out of 6 birds infected with virulent aMPV-A and aMPV-B were still aMPV positive, respectively. The virulent aMPV-A strain had a stronger impact on tissue integrity than the virulent aMPV-B strain, although collected data differed not significantly. It may be speculated that this stronger impact is the result of a longer persistence of the virulent aMPV-A strain in affected tissues than of the virulent aMPV strain.

Virulent aMPV-B showed a longer persistence and a broader tissue distribution in commercial broilers than virulent aMPV-A (AUNG et al. 2008). These observations are in contrast to those made in turkeys. Whether this difference is based on species-differences or on further factors is not known. But the differences observed for infection of turkeys with virulent aMPV-A and aMPV-B were consistent with those seen in previous studies. In these studies aMPV-A was shown to invade deeper areas of the respiratory tract, to infect two times more epithelial cells in the URT and to release higher titres of shedded virus when compared to aMPV-B (VAN DE ZANDE et al. 1999). To clarify the observed differences future studies are needed to evaluate the tissue distribution of the virulent strains of aMPV-A and aMPV-B in turkeys following infection.

6.1.2. New insights into immune reactions following aMPV-infections

Only few studies have been done on the immune reactions in turkeys after aMPV-A and aMPV-B infections. Some research was done on the humoral and CMI-related immune reactions following infection with the newly emerged aMPV subtype C. In the experiments presented here the local immune reactions and cell mediated immune reactions to aMPV infections of turkeys were evaluated.

6.1.2.1. Humoral immune reactions to aMPV

As soon as 5 to 7 days PI VN antibodies were detected locally in tracheal washes and systemically in serum independent of the strain used. VN antibody titres peaked at 7 days PI and then declined. Again at 7 to 14 days PI, the onset of serum ELISA antibodies was detected. The peak of serum ELISA antibody production varied between the groups infected with attenuated aMPV-B and virulent aMPV-A and aMPV-B. It was reached between 14 and 28 days PI.

The peak in aMPV antibodies coincided with the reduction in aMPV genome detection in nasal turbinates. Additionally, a concurrence of these parameters with the decline of microscopical lesions was found. This concurrence suggests a correlation between the stimulation of humoral immune reactions, viral clearance and lesions.

The viral clearance may be the predominant factor that allows the recovery of the respiratory mucosa (COOK et al. 1993b; MAJO et al. 1995). The results reported here support the speculation of Khehra (1998), who stated that the ELISA antibodies may play a role in the aMPV clearance. This speculation is in contrast to conclusions drawn from previous experimental and field observations indicating a minor role of ELISA antibodies in protection against aMPV field challenge (JONES et al. 1992). High levels of maternally derived antibodies as well as high levels of vaccine-induced ELISA antibodies were shown to not sufficiently protect from aMPV field infections (NAYLOR et al. 1997).

Differences were observed in the course of aMPV ELISA and VN antibody titres following vaccination or infection with different subtypes. This variation was consistent with the observations made in aMPV-challenged broilers by Aung et al. (2008). Possibly differences in subtype-specifity of the two serological tests used may explain these variations. Limited serological cross-reactivity between aMPV-A and aMPV-B was demonstrated before (HAFEZ 1992). Solely aMPV-A was used for VN antibody testing. Thus, the limited cross-reactivity may explain the conspicuous higher VN titres of aMPV-A in contrast to aMPV-B. The limited cross-reactivity may also have influenced the outcome of ELISA-titres, as the coating antigen in the commercial ELISA used was of subtype B.

6.1.2.2. Cell mediated immune reactions to aMPV

The evaluation of aMPV-infection in turkeys focussed on local CMI-related parameters. These parameters were considered to be important for the aspired challenge model for consecutive vaccination studies.

All aMPV strains induced an increase in the percentage of CD4+ T cell populations in HG at days 7 or 14 PI. Also a conspicuous infiltration of immune cells was observed microscopically in nasal turbinates and HG of aMPV infected birds. The accumulation of local CD4+ T cell subpopulations was also shown for aMPV-C infection of turkeys (SHARMA u. GERBYSHAK-SZUDY 2002). Local accumulation of immune cells in the HG was also seen before in other viral respiratory infections, such as infectious bronchitis virus (MONTGOMERY et al. 1991). It may be concluded that the local cell-mediated immune reactions play a significant role in overall immune reactivity to aMPV of the subtypes A, B and C, as stated for other pneumoviruses before (WEST et al. 1999; TEBBEY et al. 2000). The observations presented here may confirm the suggested importance of the HG for local immune reactivity in the URT (DEL CACHO et al. 1992; MASLAK u. REYNOLDS 1995).

The observations made by Jones et al. (1992) support the importance of local CMI. In his study a cyclophosphamide treatment of turkeys had no effect on the resistance to aMPV challenge following vaccination. He assumed that the observed resistance

to aMPV challenge may rather be based on CMI than on humoral immunity, which was suppressed due to cyclophosphamide.

In our experiments all aMPV strains tested induced accumulation of systemic CD4+ T cells in the spleen at 7 and 14 days PI. No explicite data on effect of aMPV-C infection on systemic T cell subpopulations have been published so far. Infection of turkeys with aMPV-C was shown to induce a transient depression of T cell mitogenesis (SHARMA et al. 2000; CHARY et al. 2002a). Nevertheless, it may be speculated that also aMPV-C induces systemic accumulation of T cells, because in the latter study the transient depression of T cell mitogenesis was followed by an increased T cell mitogenesis. This increase may result in increased systemic T cell subpopulations, as observed for CD4+ T cells in the experiments presented here.

6.1.2.3. Release of CMI-related metabolites after aMPV-infection

We evaluated the production of CMI-related bioactive metabolites after experimental infection of turkeys with attenuated aMPV-B. Attenuated aMPV-B stimulated spleen leukocytes to release significantly higher levels of total IFNs, IFNγ, IL-6 and NO in *ex vivo* culture than virus-free controls ($P < 0.05$). The upregulation of IFNs, IL-6 and NO coincided with the onset of microscopic lesions (Fig. 6.1). These findings confirm those of Chary et al. (2002b) who demonstrated increased production of NO-inducing factors (NOIF) by *ex vivo* stimulated spleen cells of aMPV-C infected birds, when compared to those of control birds. Also the transcript levels for inducible NO-synthetase (iNOS) were shown to be upregulated in spleen cells following aMPV-C infection (SHARMA et al. 2004b).

We also evaluated the effect of virulent aMPV-A and aMPV-B infections on systemic and in particular on local production of CMI-related metabolites. The infections induced an increased IFNγ expression in the HG in comparison to virus-free controls. IFNγ expression in the spleen varied between aMPV strains and days PI. These

variations may be associated to the observed differential persistence and induction of lesions of aMPV-A and aMPV-B, respectively.

With regard to the course of IFNγ transcript levels, there was a disagreement between the observations made for virulent aMPV-A and aMPV-B in the experiments presented here and the observations made for aMPV-C (SHARMA u. GERBYSHAK-SZUDY 2002; SHARMA et al. 2004b). During aMPV-C infection increased IFNγ transcript levels were consistently detectable up to 7 days PI, whereas for aMPV-A and aMPV-B no defined pattern could be observed. It is not known if these differences are based on subtype-specific differences or on differences in the technical approach of IFNγ detection.

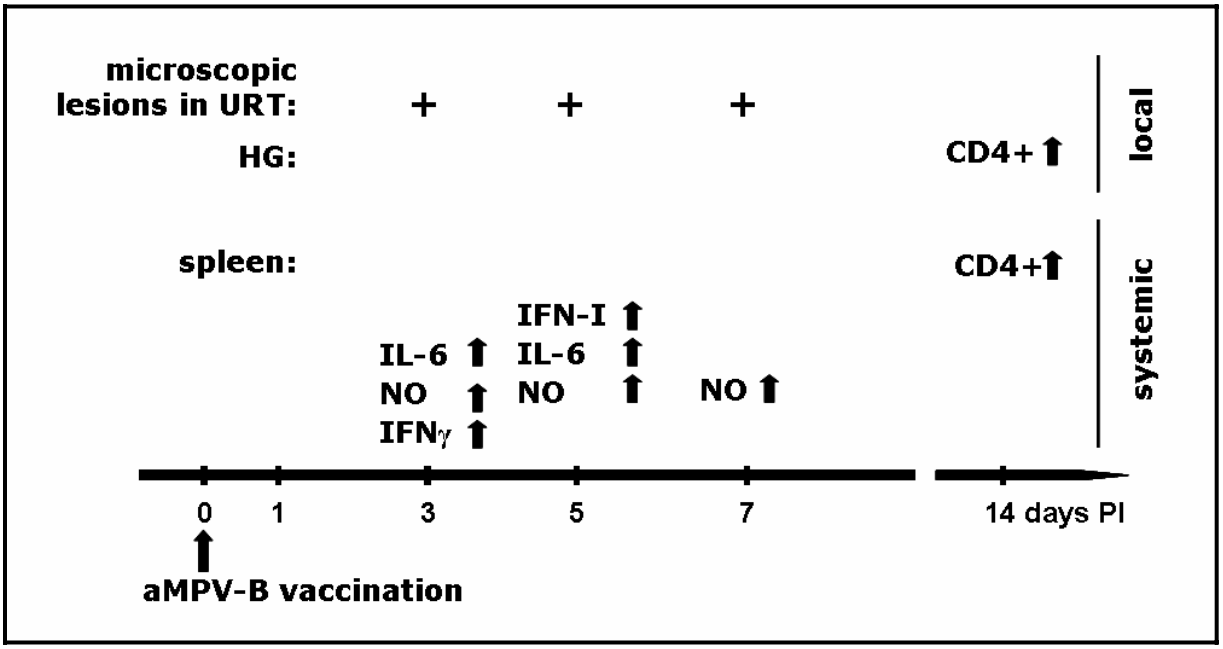

Fig. 6.1

Immune reactions to aMPV-vaccination of turkeys with an attenuated strain of subtype B.

The assessment of production of CMI-related metabolites was conducted with systemic (spleen) cells. More recently it was shown that aMPV-C antigen can be detected in peripheral organs, such as spleen (SHARMA et al. 2004b). Cook et al. (1991) isolated aMPV-A from internal organs such as heart, liver, spleen, kidney and caecal tonsils, when inoculated together with pathogenic bacteria. The production of CMI-related metabolites in spleen cells may basically be induced by the presence of aMPV antigen. Consecutively it may be concluded that the presence of aMPV antigen locally in the URT results in local production of CMI-related metabolites. This local production may influence the local immune reactions to aMPV infection in the URT.

Type I IFNs are released by virus-affected cells to activate antiviral mechanisms and NK cells (SCHULTZ et al. 2004). IFNγ is released by NK cells and Th1 cells (KAISER et al. 1998). It is involved in activation of macrophages. Activated macrophages then release IL-6 and NO (SURESH et al. 1995; QURESHI 2003). But not only macrophages, also Th2 cells produce IL-6 and induce B-cell activation (SCHNEIDER et al. 2001). Thus, all CMI-related bioactive metabolites examined may be involved in the induction of humoral as well as cellular immune reactions. They may influence the onset of microscopic lesions as well as the control of aMPV infection.

6.1.3. Correlation of evaluated parameters in aMPV pathogenesis

During the course of experimental aMPV infections we observed correlations between the analyzed parameters depending on the phase of infection.

During the acute phase of infection up to 7 days PI with virulent aMPV-A and aMPV-B the following observations correlated:

- severest clinical signs,
- most prominent microscopical lesions,
- significantly increased percentages of CD4+ T cells in HG and spleen,
- upregulation of IFNγ expression and

- detection of aMPV genome in nasal turbinates in all birds tested.

In the later phase of aMPV infection past 7 days PI the following parameters correlated:

- termination of apparent clinical signs,
- beginning of mucosal regeneration,
- peak of ELISA and VN antibody production,
- decrease in microscopically observed accumulation of immune cells and
- decline of relative number of local CD4+ T cells following transient accumulation of local and systemic CD4+ T cells.

6.1.4. Challenge model for vaccine testing

The stimulation of serum virus-neutralizing antibodies and of the local immune reactions was found to be temporary. The temporary character of the immune reactions may explain the short duration of protection against challenge following aMPV vaccination in the field. This temporary character of immune reactions after live viral vaccination has to be considered when designing a new vaccination strategy. The induction of a long-lasting protective immunity should be one of the major goals.

The observations presented here demonstrate that the analyzed humoral and cell mediated immune parameters in aMPV-A and aMPV-B infection are comparable to those described for aMPV-C infections. It was concluded that the pathogenesis of aMPV infections is based on the same mechanisms. Nevertheless, the extensive evaluation of a variety of parameters with respect to pathogenesis, local immune reactions and CMI revealed slight differences between the virulent aMPV subtypes A and B.

Particular parameters, such as the relative number of CD8+ T cells or IFNγ transcript levels in the HG and the spleen, did not yield significant and consistent data. Other parameters, such as *ex vivo* production of CMI-related bioactive metabolites by

spleen cells, were considered to be too time consuming and laborious with respect to experimental setup, laboratory space and staff.

Thus, it was decided to use aMPV-A in the consecutive experimental testing of a new vaccination strategy. The aMPV-A challenge model was limited to assessment of the following parameters:

- clinical signs
- microscopic lesions in the URT
- detection of aMPV genome in choanal swabs
- serum VN and ELISA antibodies
- CD4+ and CD8+ T cell populations in HG and spleen.

6.2. Development of a new generation aMPV-vaccine

A prime-boost vaccination strategy based on genetically engineered vaccines and PLGA-MP was developed. The goal was to induce a long-lasting and protective, local immunity against aMPV infection in turkeys.

6.2.1. Preparation and characterization of vaccine components

The new generation vaccination strategy was comprised by repeated priming with a F protein encoding DNA vaccine bound to PLGA-MP and by booster vaccination with a homologous recombinant F protein vaccine encapsulated in PLGA-MP. The PLGA-MP served as a controlled release system. The DNA vaccine, the recombinant protein vaccine, the PLGA-MP and also the resulting vaccine-loaded MP were evaluated *in vitro* and *in vivo* in chicken cells and commercial turkeys, respectively.

6.2.1.1. F protein-encoding DNA vaccine

Following cloning, the F protein encoding plasmid was transfected to CEFs. Typical syncytial formation (BUYS et al. 1989a) revealed expression of a functional F protein

in eukaryotic cells. MP-bound DNA vaccine was given *in vitro* to primary chicken macrophages and also to the chicken mononuclear cell line MQ-NCSU (QURESHI et al. 1990). The macrophages rapidly took up the MP. Consecutively, the MP as well as expressed F protein were detectable in the cells. As shown in mammalian cells, the incubation with and the uptake of PLGA-MP did not alter the cells viability or death rate (WALTER et al. 2001). This is the first study that demonstrates the successful *in vitro* uptake and subsequent expression of a MP-bound DNA vaccine by avian cells. The *in vitro* uptake and expression of plasmid DNA bound to or encapsulated in MP has only been shown in mammalian cells before (DENIS-MIZE et al. 2000; WALTER et al. 2001; SHARPE et al. 2003).

In a complementary pre-study the naked F protein-encoding DNA vaccine was repeatedly inoculated intramuscularly to SPF chickens. In this pre-study two out of three inoculated birds developed aMPV-specific VN antibodies in contrast to no development of aMVP-specific VN antibodies in non-treated control birds. Thus, the DNA vaccine was shown to be immunogenic *in vivo*. Plasmid-encoded F protein of subtype C was shown to be immunogenic *in vivo* before (TARPEY et al. 2001; KAPCZYNSKI u. SELLERS 2003).

6.2.1.2. Recombinant F protein vaccine

Recombinant F protein was characterized following SDS-PAGE and transfer to a PVDF membrane by Western blotting. The F protein bands were specifically detected by binding of immunoglobulins purified from reconvalescent's sera of aMPV-A infected chickens. Specifically detected bands of recombinant F protein were compared to those of control virus propagated in cell culture and to the molecular weight standards. Based on these results and with respect to the characteristics of aMPV F protein described before (CAVANAGH u. BARRETT 1988), the successful production of F protein precursor F_0 and subunits F_1 and F_2 was concluded.

6.2.1.3. PLGA-MP

A protocol for preparation of cationic MP was established. The cationic MP were designed to be a controlled release system for the DNA vaccine. The produced MP were shown to have the aspired size of up to 10 µM. MP of this size were shown to efficiently be taken up and consecutively be processed by phagocyting cells (PORTER et al. 1997; HEDLEY 2003; PEYRE et al. 2004). As shown before, the DNA vaccine was efficiently bound to the cationic MP-surface. No adverse effects on the structural integrity of the DNA during preparation and during subsequent *in vitro* incubation over time was observed, as shown before (HE et al. 2005). The bound plasmid DNA was shown to efficiently be protected by the carrier system against degradation by DNAse I, as demonstrated before (OSTER et al. 2005). It was concluded that the cationic MP may also efficiently protect the DNA vaccine against degradation by ubiquitous nucleases *in vivo* and release sufficient amounts of functional DNA vaccine.

The protocol for the encapsulation procedure of recombinant F protein was established. The resulting MP were characterized for size distribution and total protein loading. The size distribution was comparable to that of the cationic MP. But the total protein load was low in comparison to encapsulated protein concentrations published before (IGARTUA et al. 1998; GUTIERRO et al. 2002). The reason for this low loading is not known. It may be speculated that the strong hydrophobicity and thus low solubility of the F protein interferes with the efficient encapsulation in the water-in-organic-solvent-in-water homogenization process. Additionally, the protein-quantitation assay may have given misleading results due to the tendency of the recombinant F protein to precipitate in concentrated solutions.

The *in vitro* and *in vivo* results indicate that the MP-bound DNA vaccine construct and the MP-encapsulated recombinant F protein construct may be applicable to poultry and may induce aMPV-specific immune reactions. It may be assumed that both MP-vaccines efficiently are taken up from conjunctival and respiratory mucosa and consecutively are processed for MHC antigen presentation (FIX u. ARP 1991;

HOSHI et al. 1999). The antigen presentation of immunogenic F protein is speculated to induce broad local immune reactions.

6.2.2. *In vivo* testing of the new generation vaccination strategy

The developed vaccines were tested *in vivo* with regard to safety, induction of immune reactions and protective power against aMPV challenge infection.

6.2.2.1. Safety and induction of immune reactions in turkeys

The safety and immunogenicity of the applied vaccination system was evaluated during the steps of prime-boost immunization and up to 14 days post last vaccine application.

The oculonasal application of the vaccines did not induce any irritation of mucosal surfaces. It did not induce any clinical signs, macroscopic or microscopic lesions. A conspicuous infiltration of the nasal turbinates and HG with immune cells at 8 days post booster vaccination was observed microscopically solely in the F protein-specific immunized birds. This infiltration was confirmed by the observation of increased CD4+ T cell populations locally in the HG at 13 days post booster vaccination in both experiments. Increased CD4+ T cell populations were also found systemically in the spleen at 8 days post booster vaccination.

The F protein-specific immunization did not induce production of significant aMPV-specific ELISA antibody titres. But the F protein-specific immunization induced the production of aMPV-specific VN antibodies. In non-immunized control birds maternally derived VN antibodies were detectable up to 8 days post booster vaccination. In contrast to that aMPV-specific VN antibodies were detected up to the end of the experiment at 21 days post last booster vaccination in F protein-specific immunized birds. The VN antibody titres detected in sera of F protein-specific immunized birds were higher throughout the whole experiment when compared to the non-immunized control birds (significant higher at day of second MP-vaccination;

$P < 0.05$). More F protein-specific immunized birds per group were VN antibody positive throughout the whole experiment when compared to the non-immunized control group.

6.2.2.2. Protection provided by the vaccination

For evaluation of the protective power of the F protein-specific immunization the previously defined challenge model was applied.

The clinical signs after challenge with virulent aMPV-A were significantly reduced in F protein-specific immunized birds ($P < 0.005$). Interestingly, mock-loaded MP-immunized control birds also showed less clinical signs than the non-vaccinated and challenged control birds ($P < 0.05$). The development of severe clinical signs was delayed in F protein-specific immunized birds as compared to non-vaccinated and challenged control birds. Additionally, the challenged, F protein-specific immunized birds also recovered faster than the non-vaccinated control birds did.

The microscopic lesions induced by the challenge infection were comparable to those seen in the pathogenesis studies of aMPV. F protein-specific immunization, mock-loaded MP immunization and live viral vaccination provided partial protection against microscopical lesions when compared to non-vaccinated challenged control birds. The reduction of lesions was most distinct in the F protein-specific immunized birds. But also non-specific vaccination with mock-loaded MP led to reduced lesions after challenge. These non-specific effects of mock-loaded MP immunization may be due to adjuvant effects or other non-specific immunomodulating effects of the PLGA-MP and/ or the encapsulated heterologous protein BSA.

The effects of immunizations on local immune cell infiltration could not be compared between groups due to the microscopic lesions in the URT following challenge. The effect of the aMPV challenge on local CD4+ T cells was comparable to that observed following vaccination in non-challenged birds. Thus, the challenge infection did not significantly increase the accumulation of CD4+ T cells as compared to F protein-

specific immunized, non-challenged birds. This lack of effect may be due to a transient depression of the T cell mitogenesis caused by the virulent challenge strain (CHARY et al. 2002b). Contrary to that, an increased accumulation of systemic CD4+ T cells was detected in spleen of challenged, F protein-specific immunized birds as compared to non-challenged, F protein-specific immunized birds. It may be concluded that the accumulation of systemic CD4+ T cells is supported by the challenge infection.

The effect of F protein-specific vaccination on virus shedding following challenge was investigated by nested RT-PCR based detection of aMPV in choanal swabs. In all vaccinated groups the number of aMPV positive birds was reduced following challenge, when compared to non-vaccinated, challenged control birds. In the F protein-specific vaccinated group and in the group vaccinated with a commercial live vaccine was no detection of aMPV after 11 and 5 days PI, respectively. In mock-loaded MP vaccinated and non-vaccinated, challenged control groups challenge virus was detected until the end of the experiments. Thus, F protein-specific immunization reduced virus release when compared to the control groups.

The aMPV challenge induced production of ELISA-detectable antibodies. But no effect of F protein-specific immunization on the ELISA titres could be observed as compared to non-vaccinated, challenged control birds. At 13 days post challenge all birds in all challenged groups were VN antibody positive. The F protein-specific immunized birds were shown to have significant higher VN antibody titres when compared to non-vaccinated, challenged birds ($P < 0.05$). Whether the reduced virus excretion in F protein-specific immunized birds is due to the VN antibody response or due to aMPV-specific cell mediated immunity is not known.

In summary, the F protein-specific immunization provided partial protection against challenge with virulent aMPV. The F protein-specific immunization

- induced no adverse effects on the URT *in vivo*;
- reduced clinical signs;

- reduced microscopic lesions;
- induced (microscopically observed) infiltration of affected tissues with immune cells;
- shortened viral shedding;
- induced accumulation of local and systemic CD4+ T cells;
- induced production of systemic aMPV-neutralizing antibodies;

in comparison to the mock-loaded MP-vaccinated or non-vaccinated, challenged birds.

6.2.2.3. Unspecific effects of mock-microparticle application

It may be speculated that effects specifically induced by F protein-specific immunization, such as VN antibody production and enhanced aMPV-specific CMI, as well as non-specific adjuvant effects may have contributed to the observed partial protection. These non-specific adjuvant effects have been reported before for PLGA-MP and for the CpG-motifs in the plasmid DNA vaccine (IGARTUA et al. 1998; WALTER et al. 2001; KRIEG 2002; SUN et al. 2003; OSTER et al. 2005). In the experiments presented here those non-specific effects interfered with the demonstration of aMPV-specific protective effects, as non-specific and specific effects on distinct parameters could not be differentiated.

The non-specific effects may play an important role in the overall stimulation of the non-specific and specific immune reactions provided by such vaccination strategy. The induced non-specific “first line of defence” mechanisms may provide some protective effects in the period between vaccination and onset of specific protective immunity.

6.3. Conclucions and future perspectives

Extensive experimental studies on the pathogenesis and immune reactions to aMPV-A and aMPV-B infections in turkeys were conducted. These studies were the first with explicit focus on CMI-related parameters in the pathogenesis of European aMPV strains. The results indicated that the CMI-related immune reactions are widely involved in the immune reactivity to aMPV infections. In principle, the investigated parameters of aMPV pathogenesis showed comparable results between the applied virus strains of different subtype and virulence.

These studies helped to understand more about the pathogenesis and the immune reactions to aMPV infection. But still open questions are left. Further studies are needed to explore the immune reactions involved in induction of lesions as well as in protection against aMPV infection in more detail. Particularly, the better understanding of the contribution of local, virus-specific T cell immunity to protective effects may be of interest. To evaluate this, further *in vitro* techniques have to be established, such as an assay for the measurement of aMPV-specific T cell reactivity.

The pathogenesis studies enabled the development of an aMPV challenge model in turkeys. The parameters that were evaluated in the challenge model may also be useful for the establishment of challenge models for further pathogens that affect the URT of avian species.

This challenge model was subsequently used to test a new vaccination strategy that was experimentally applied to turkeys. This concept was supposed to overcome today's problems with vaccination of turkeys with current commercial aMPV vaccines. These studies were the first to apply an oculonasally MP-delivered, genetically engineered vaccination regime in the avian system.

The applied vaccination strategy only induced partial protection. Thus, further improvement of the vaccination strategy needs to be done. To improve the vaccination strategy, the vaccine components should be optimized:

- The DNA vaccine may be optimized for host codon usage (MENNUNI et al. 2005).
- The protein encoding DNA vaccine as well as the recombinant protein vaccine may be optimized with respect to directed stimulation of T cells by respective designed epitopes (PLOTNICKY-GILQUIN et al. 2002).
- The recombinant protein is suggested to be of disadvantageous high hydrophobicity. Thus, the recombinant protein may be optimized by elimination of respective hydrophobic aa-sequence segments.
- The MP's surface characteristics and size may be modulated to improve the stimulation of the MP-uptake by phagocytic cells.
- The MP's vaccine loading and release may be improved.
- The schedule of vaccine application may be modulated to improve booster effects.

A sufficient improvement of this vaccination system may allow for *in vivo* application of this strategy for the protection against single or even multiple, economically important respiratory or enteric pathogens in poultry. The vaccination strategy may also be tested in further avian and mammalian species.

7. Summary

Martin Liman

Studies on the pathogenesis of avian Metapneumovirus (aMPV) infection in turkeys and on the protective effect of a microparticle-based, genetic engineered aMPV vaccination strategy

Avian Metapneumovirus (aMPV) infections have a significant economical impact on commercial poultry production. Despite the use of commercial live and inactivated vaccines field outbreaks with respiratory disease (turkey rhinotracheitis, TRT) often occur. The virus may also cause transient immunosuppression. Field outbreaks are often exacerbated by secondary bacterial infections of the upper respiratory tract.

The goal of this project was to develop a new prime-boost vaccination concept against aMPV infection. To improve aMPV vaccination strategies it was necessary to establish an aMPV challenge model. A specific focus was placed on the investigation of local immune mechanisms, which may play an important role in protection against the infection.

Two studies on the pathogenesis and immune reactions to attenuated and virulent aMPV strains of subtypes A and B were conducted. The investigated parameters of aMPV pathogenesis showed comparable results between the virus strains of different subtype. It was demonstrated that humoral and CMI-related immune reactions were induced not only systemically but also locally after infection with the different aMPV strains. Future studies are needed to give deeper insights into the role of local T cell-mediated immune reactions in protection against aMPV.

The newly developed vaccination strategy was comprised by genetically engineered vaccines combined with microparticles (MP). A DNA vaccine encoding for the fusion

(F) protein of aMPV, and the homologous recombinant F protein were chosen to be the most promising candidates for an oculonasally applied prime-boost immunization. The goal was to induce a broad humoral and cellular immunity for long-lasting protection against aMPV infection. MP were used as a controlled release system for the genetically engineered vaccines, and protected the sensitive vaccine components against degradation *in vivo*. Thus, the system could be used for mass application to commercial poultry via spray, drinking water or feed. The new vaccination strategy was tested for safety and immunogenicity *in vitro.* Consecutively it was tested for safety, induction of immune reactions and protection in the established aMPV-challenge model in commercial turkeys. The applied vaccination strategy was shown to not induce adverse effects, and to stimulate local and systemic, humoral and cellular immune reactions. It induced partial protection against aMPV challenge.

These were the first studies that applied an oculonasally delivered, genetically engineered vaccination regime in the avian system. Based on these investigations future studies have to be conducted to improve this genetically engineered vaccination strategy.

8. Zusammenfassung

Martin Liman

Studien über die Pathogenese der aviären Metapneumovirus (aMPV) Infektion der Pute und über die Schutzwirkung einer Mikropartikel (MP)- basierten und gentechnisch hergestellten aMPV-Impfstrategie

aMPV Infektionen spielen eine wirtschaftlich bedeutende Rolle in der Geflügelproduktion. Trotz Impfung mit kommerziellen Lebend- und Inaktivatvakzinen kommt es häufig zu Feldausbrüchen mit respiratorischer Erkrankung (Puten-Rhinotracheitis, turkey rhinotracheitis, TRT). aMPV kann eine vorübergehende Immunsuppression bewirken. Feldausbrüche werden häufig von schweren bakteriellen Sekundärinfektionen begleitet.

Ziel dieses Projektes war es, ein neuartiges „prime-boost“ Impfkonzept zum Schutz vor aMPV Infektionen zu entwickeln. Dazu wurde zunächst ein aMPV-Belastungsmodel etabliert. Es wurden insbesondere die lokalen Immunmechanismen untersucht, da man einen wesentlichen Beitrag dieser an der schützenden Immunität annimmt.

Es wurden sowohl attenuierte als auch virulente aMPV-Isolate der Subtypen A und B hinsichtlich der Pathogenese und der Immunreaktionen auf die Infektion untersucht. Die Ergebnisse zu den untersuchten Parametern der aMPV-Pathogenese waren vergleichbar zwischen den Isolaten verschiedener Virulenz und Subtypen. Es konnte gezeigt werden, dass die Infektionen mit den verschiedenen Isolaten nicht nur systemische, sondern auch lokale humorale und zellvermittelte Immunreaktionen hervorrufen. Es sind aber weitere Studien nötig, insbesondere um die Rolle der lokalen T-Zell-vermittelten Immunreaktionen für die zu erzielende Schutzwirkung weiter zu klären.

Das entwickelte neuartige Impfkonzept basierte auf gentechnisch hergestellten Impfstoffen, die mit Mikropartikeln kombiniert wurden. Ein für das Fusion- (F) Protein kodierender Plasmid-Impfstoff sowie das dazu homologe rekombinante F Protein wurden für eine okulonasal zu applizierende „prime-boost“ Impfstrategie hergestellt. Ziel des Impfkonzeptes war es, eine belastbare und dauerhafte humorale und zelluläre Immunität zu bewirken. Die MP wurden zusätzlich eingesetzt, da sie die gentechnisch hergestellten Impfstoffe, die *in vivo* schnell zerstört werden, schützen und kontrolliert freisetzen. Auf diese Weise ist das Impfkonzept auch für eine Massenimpfung von Geflügel durch Versprühen oder durch Applikation über das Trinkwasser oder das Futter geeignet. Die Impfstoffe wurden zunächst *in vitro* auf die biologische Unbedenklichkeit und eine immunogene Wirkung untersucht. Anschließend wurde das Impfkonzept bei kommerziellen Puten okulonasal angewandt und auf seine Unbedenklichkeit, auf seine immunstimulierende Wirkung und auf die erzielte Schutzwirkung untersucht. Die Impfstoffe und das Impfkonzept hatten keine unerwünschten Wirkungen, haben aber die Puten zu lokalen und systemischen, humoralen wie zellulären Immunreaktionen stimuliert. Das Impfkonzept hat eine teilweise Schutzwirkung erzielt.

In diesen Impfstudien wurde das erste Mal ein Impfkonzept, das auf der lokalen Verabreichung von gentechnisch hergestellten und mit MP kombinierten Impfstoffen basiert, bei Geflügel angewandt. Für weitere Studien sollte die Wirkung der gentechnisch hergestellten Impfstoffe und die Herstellung der MP optimiert werden.

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