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Recombinant Marek's disease virus (MDV) lacking the *Meq* oncogene confers protection against challenge with a very virulent plus strain of MDV

Lucy F. Lee[a,*], Blanca Lupiani[b], Robert F. Silva[a], Hsing-Jien Kung[c], Sanjay M. Reddy[b]

[a] *Avian Disease and Oncology Laboratory, Agricultural Research Service, East Lansing, MI 48823, United States*
[b] *Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843, United States*
[c] *Department of Biological Chemistry, School of Medicine, University of California, Davis, UC Davis Cancer Center, Sacramento, CA 95817, United States*

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Summary Marek's disease virus (MDV) encodes a basic leucine-zipper protein, *Meq*, that shares homology with the Jun/Fos family of transcriptional factors. Conclusive evidence that *Meq* is an oncogene of MDV came from recent studies of a Meq-null virus, rMd5△Meq. This virus replicated well in vitro, but was non-oncogenic in vivo. Further characterization of this virus in vivo indicated that the *meq* gene is dispensable for cytolytic infection since it replicated well in the lymphoid organs and feather follicular epithelium. Since rMd5△Meq virus was apathogenic for chickens, we set out to investigate whether this virus could be a good candidate vaccine. Vaccine efficacy experiments conducted in Avian Disease and Oncology Laboratory (ADOL) $15I_5 \times 7_1$ chickens vaccinated with rMd5△Meq virus or an ADOL preparation of CVI988/Rispens indicated that the Meq-null virus provided protection superior to CVI988/Rispens, the most efficacious vaccine presently available, following challenge with a very virulent (rMd5) and a very virulent plus (648A) MDV strains.
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Introduction

Marek's disease virus (MDV) is a member of the genus *Mardivirus*, sub-family *Alphaherpesvirinae* in the family *Herpesviridae*. MDVs are classified into three closely related but distinct groups. Serotype 1 viruses (GaHV-2) cause an acute lymphoproliferative disease in chickens, resulting in T-cell lymphomas that metastasize to visceral organs and

* Corresponding author. Tel.: +1 517 337 6836; fax: +1 517 337 6776.
E-mail address: lucy.lee@ars.usda.gov (L.F. Lee).

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peripheral nerves. Serotypes 2 (GaHV-3) and 3 viruses (MeHV-1) are nonpathogenic and were isolated from chickens and turkeys, respectively.

Vaccines have been cornerstones in the control of Marek's disease (MD) in chickens [1,2]. The first vaccine used, HPRS-16/att, was derived by attenuation of a virulent strain and was introduced in 1969 [3,4]. This vaccine was quickly replaced by a turkey herpesvirus (HVT), an antigenically related virus belonging to serotype 3, which had better replication in chickens [5,6]. The increased virulence of field isolates in vaccinated chickens led to the use of bivalent vaccines consisting of HVT virus along with serotype 2 strains like SB-1 or 301B/1 in 1983 [7,8]. Though the mechanism of MD protection is poorly understood, it was clear that a synergistic protective effect was obtained when bivalent vaccines were used [9].

The use of vaccines to control MD is suggested to have led to the evolution of the field viruses towards greater virulence [10]. Therefore, in the early 1990s, the serotype 1 CVI988/Rispens vaccine was introduced in the United States [11]. CVI988/Rispens was originally isolated in 1972 (prior to the widespread use of vaccines) in The Netherlands and was shown to be a mildly virulent serotype 1 virus. This virus was further attenuated by cell culture passage to generate a vaccine able to confer protection superior to that of bivalent vaccines against highly virulent MDV strains [12,13].

At present, CVI988/Rispens virus is used worldwide for controlling MD caused by very virulent plus (vv+) strains and no better vaccines are currently available. The continued evolution of MDV towards greater virulence has prompted concern that the currently available vaccines will ultimately loose efficacy in controlling MD [14]. This led several investigators to develop more efficacious vaccines, but it has been a difficult challenge. Recently, Witter and Kreager [15] compared 10 strains of vaccine viruses and none showed a better protection against the disease than the CVI988/Rispens virus. They concluded that conventional vaccine development may have approached biological threshold of vaccine efficacy and therefore, new strategies are needed for vaccine development. Recombinant DNA technology has aided in the development of novel vaccines. Fowlpox vector vaccines designed to express several MDV envelope glycoproteins proved efficacious in protecting MD in chickens under laboratory testing [16—19]. However, these vaccines are not commercially used because they do not confer superior protection when compared to CVI988. Moreover, the presence of maternally derived antibodies to fowlpox, will greatly suppress the vaccine efficacy of fowlpox-vectored vaccines. A full length MDV BAC derived DNA vaccine formulation was shown to confer some degree of protection [20]. The BAC DNA was derived from a cell culture attenuated strain of a very virulent plus MDV 584A strain [10]. The full length genomic BAC DNA, when injected into muscle tissue resulted in the reconstitution of an infectious virus. These DNA vaccine formulations may have some use in the future, but as of now they do not have superior efficacy compared to commercially available vaccines.

The development of both cosmid DNA and BAC technologies has greatly facilitated the introduction of mutations into the viral genomes to study gene functions. Using these technologies, the function of several MDV genes has been investigated. We and others have shown that the genes present in the long repeat regions of the MDV genome, including viral telomerase RNA [21,22], viral IL-8 [23-26], *Meq* [27,28], pp38 [29,30], and RLORF4 [31] play an important role in pathogenesis. Among these genes, only *Meq* has been shown to be consistently expressed in all MDV tumor and latent cells and is only present in serotype 1 strains but not in non-oncogenic serotypes 2 and 3 of MDV. Meq is a 339-amino acid long protein encoded within the MDV EcoRI Q fragment of serotype 1 [32]. There are two copies of *Meq* in the MDV genome, one in each of the repeat long regions (TR_L and IR_L).

We have previously shown that deletion of the *Meq* gene resulted in loss of transformation of T-cells in chickens, but had no effect on the early cytolytic phase of infection in the lymphoid organs [27]. Since the *Meq* null virus was apathogenic for chickens, we set out to investigate whether this virus would be a good candidate vaccine. Vaccine efficacy experiments conducted in MDV maternal antibody positive and negative Avian Disease and Oncology Laboratory (ADOL) $15I_5 \times 7_1$ chickens vaccinated with rMd5△Meq virus or an ADOL preparation of CVI988/Rispens indicated that the *Meq* null virus provided protection superior to that of CVI988/Rispens following challenge with the vv+ 648A strain.

Materials and methods

Cells and viruses

Primary duck embryonic fibroblasts (DEF) were used for virus propagation and virus reactivation assays. The reference serotype 1 vaccine virus CVI988/Rispens strain was provided to ADOL by Merial Select [13] and vaccine stocks were prepared in this laboratory. Recombinant rMd5△Meq virus, which lacks the meq oncogene, and wild-type recombinant Md5 virus (rMd5) were generated from cosmids derived from the very virulent (vv) Md5 strain as previously described [30]. vv rMd5 and very virulent plus (vv+) 648A strains of serotype 1 MDV [10,33] were used as challenge viruses.

Chickens

For laboratory experiments, chickens were F_1 progeny of line $15I_5$ males and line 7_1 females. For some experiments, these were from breeder hens free of maternal antibody (ab−) while for others the breeder hens were vaccinated with all three MD vaccine serotypes and were considered positive for maternal antibodies (ab+). All breeder chickens were maintained at the ADOL and were free of antibodies to avian leukosis virus, reticuloendotheliosis virus and various other poultry pathogens.

Vaccine experiments

To study the protection efficacy of rMd5△Meq in the laboratory setting, 17-day-old ab+ or ab− $15I_5 \times 7_1$ chicks were vaccinated with 2000 plaque-forming-units (PFU) of

Table 1 Protective efficacy of rMd5△Meq against vv+ 648A virus challenge in MDV maternal antibody negative (ab−) chickens

Vaccine	Replicate 1			Replicate 2			Summary		
	MD mort.	MD/total (%)	PI	MD mort.	MD/total (%)	PI	MD mort.	MD/total (%)	PI[a]
rMd5△Meq	0/15 (0)	0/15 (0)	100	0/17 (0)	0/17 (0)	100	0/32 (0)	0/32 (0)	100 a
CVI988/Rispens	7/14 (50)	7/14 (50)	50	5/17 (29)	5/17 (29)	71	12/31 (39)	12/31 (39)	61 b
None	17/17 (100)	17/17 (100)	0	13/13 (100)	13/13 (100)	0	30/30 (100)	30/30 (100)	0 c

MD mort. = Marek's disease mortality; % MD = % Marek's disease; PI = protection index.
[a] PI among the three experimental groups with different lowercase letters differ significantly based upon χ^2 analysis ($p < 0.05$).

rMd5△Meq or CVI/988/Rispens vaccine virus by the intra-abdominal (IA) route. Five days later, groups of vaccinated and unvaccinated control chickens were challenged by IA inoculation with 500 PFU of vv rMd5 or vv+ 648A MDV. To determine the effect of vaccination on challenge virus load, chickens were bled at 1, 3 and 7 weeks post-challenged and virus load was determined by reisolation of virus from buffy-coats as indicated below. Mortality during the course of the experiment was recorded and chickens were examined for gross MD lesions. At about 56 days post-challenge, all surviving chickens were euthanized and examined for gross MD lesions. The percentage of gross MD was calculated for each test group as the number of chickens with gross MD lesions divided by number at risk (survivors + MD deaths) × 100. Vaccinal immunity to MD was expressed as a protective index (PI) calculated as the percent gross MD in non-vaccinated challenged control chickens minus the percentage of gross MD in vaccinated, challenged chickens divided by the percentage of gross MD in non-vaccinated challenged control chickens × 100.

Virus isolation from vaccinated and challenged chickens

Blood was collected from vaccinated and challenged ab− chickens in the presence of heparin at different times post-challenge and buffy-coat cells were obtained by centrifugation. Lymphocytes from the buffy-coats were counted, diluted to 10^6 cells/ml and duplicated 35-mm plates of freshly seeded DEF monolayers infected with 10^6 lymphocytes for each chicken sample. To determine viremia levels, visible viral plaques were counted 5–6 days post-infection.

Results

Vaccine experiments

The protection efficacy of rMd5△Meq and CVI988/Rispens to challenge with the vv+ virus 648A was compared in ADOL $15I_5 \times 7_1$ ab+ and ab− chickens. All the ab+ and ab− chickens in the non-vaccinated and challenged group showed 100% MD specific mortality and lesions, whereas none were observed in either group of chickens vaccinated with rMd5△Meq (Tables 1 and 2). On the other hand, the CVI988/Rispens vaccinated and challenged chickens showed 39 and 41% MD in ab− and ab+ chickens, respectively (Tables 1 and 2). The protective index of the two vaccines used was 100 and 61 for ab− and 100 and 59 for ab+, for rMd5/△Meq and CVI988/Rispens, respectively.

Similar experiments were carried out in ab− $15I_5 \times 7_1$, but the vaccinated chickens were challenged with two virulent pathotypes, the vvMDV strain, rMd5 and the vv+MDV strain, 648A. The rMd5△Meq virus protected 100% of chickens upon challenge with both viral strains whereas CVI988/Rispens protected 100% with rMd5 and 87.5% with 648A (Table 3). Therefore, based on the MD incidence, the protection index of rMd5△Meq was 100% in both virus challenges while the protection index of CVI988/Rispens was 100 and 87.5 in rMd5 and 648A, respectively.

Effect of vaccination on the level of MDV viremia

Groups of 1-day-old chicks were vaccinated with rMd5△Meq or CVI988/Rispens or were unvaccinated. At 1 week post-vaccination, all chickens were challenged with 500 PFU of rMd5. Five chickens from each group were bled at 1, 3, and 7

Table 2 Protective efficacy of rMd5△Meq against vv+ 648A virus challenge in MDV maternal antibody positive (ab+) chickens

Vaccine	Replicate 1			Replicate 2			Summary		
	MD mort.	MD/total (%)	PI	MD mort.	MD/total (%)	PI	MD mort.	MD/total (%)	PI[a]
rMd5△Meq	0/17 (0)	0/17 (0)	100	0/17 (0)	0/17 (0)	100	0/34 (0)	0/34 (0)	100 a
CVI988/Rispens	5/17 (29)	7/17 (41)	59	NT	NT	NT	5/17 (29)	7/17 (41)	59 b
None	10/15 (66)	15/15 (100)	0	14/15 (93)	15/15 (100)	0	24/30 (80)	30/30 (100)	0 c

MD mort. = Marek's disease mortality; % MD = % Marek's disease; NT = not tested due to mortality as result of the flood in the isolator; PI = protection index.
[a] PI among the three experimental groups with different lowercase letters differ significantly based upon χ^2 analysis ($p < 0.05$).

Table 3 Protection of rMd5△Meq and CVI988/Rispens viruses in MDV maternal antibody negative (ab−) chickens against challenge with two MDV pathotypes

Vaccine	Challenge virus	MD mortality (%)	MD (%)	PI[a]
rMd5△Meq	Md5	0/15 (0)	0/15 (0)	100 a
	648A	0/17 (0)	0/17 (0)	100 a
CVI988/Rispens	Md5	0/15 (0)	0/15 (0)	100 a
	648A	2/16 (12.5)	2/16 (12.5)	87.5 b
None	Md5	17/17 (100)	17/17 (100)	0 c
	648A	13/13 (100)	13/13 (100)	0 c

% MD = % Marek's disease; PI = protection index.
[a] PI among the three experimental groups with different lowercase letters differ significantly based upon χ^2 analysis ($p < 0.05$).

Table 4 Reduction of viremia in MDV maternal antibody negative (ab−) chickens vaccinated and challenged with vv rMd5 virus[a]

Vaccine	Weeks post-challenge					
	1		3		7	
	PFU	% reduction	PFU	% reduction	PFU	% reduction
None	330		149		258	
rMd5△Meq	99	70	2	98.7	0	100
CVI988/Rispens	21	93.6	30	79.9	4	98.4
None/no challenge	0		0		0	

[a] Chicks were vaccinated at day of age with 2000 PFU of the indicated vaccines and challenged 5 days later with 500 PFU of vv rMd5 virus.

weeks post-challenge, and cell-associated MDV viremia was determined. As shown in Table 4, both of the vaccines caused a significant and sustained reduction in the level of MDV viremia in vaccinated chickens throughout the experiment. At 1 week after challenge with rMd5, the unvaccinated group had a viremia titer of 330 PFU/1 $\times$ 10^6 buffy-coat cells whereas the rMd5△Meq and ADOL CVI988/Rispens vaccinated groups had 99 and 21 PFU, respectively which were significantly different from each other and also from the none vaccinated group. The reduction of MDV level at 1 week post-challenge for rMd5△Meq and CVI988/Rispens was 70 and 93.6%, respectively. At 3 weeks post-challenge, the per-cent reduction of viremia for these two viruses was 98.7 and 79.9, respectively, while at 7 weeks, the per-cent of reduction was 100 and 98.4%, respectively. These results show that both vaccines protect against MD and also significantly reduce replication of the challenge virus.

Discussion

MDV is a highly contagious herpesvirus, which elicits a rapid onset of malignant T-cell lymphomas in chickens, usually within weeks after infection. Vaccines have become an important means of control of MD in the field since their introduction in 1969. With the widespread use of vaccines there has been an increase in virulence of MDV field strains. The decline of the protective efficacy of HVT and bivalent vaccines, probably due to the emergence of very virulent strains, prompted concerns that all currently available vaccines will eventually become less protective [14]. These concerns have stimulated research into development of improved vaccines to protect newly emerging highly virulent MDV field isolates. The production of improved vaccines has been a challenging task, with no commercially viable product developed since the introduction of bivalent vaccines in the 1980s. Several potential vaccines have been experimentally generated but are less efficacious than currently available vaccines [34—36]. The vaccines that are highly efficacious lacked the safety profile necessary for commercial use [25,33]. These observations suggest that we may be approaching the threshold of vaccine efficacy to MD [15].

Our laboratory and others have used a scientific method to identify genes involved in pathogenesis in order to generate improved vaccines to control MD. Meq is the only protein persistently expressed in MDV tumors and MDV transformed T-lymphoblast cell lines [32] and thus is likely to play a regulatory role in transformation and latency. We have previously described a *Meq* null virus, rMd5△Meq, in which both copies of the *Meq* gene had been deleted. In vivo studies with ab− chickens indicated that the rMd5△Meq virus was attenuated, resulting in no incidence of gross or microscopic lesions and mortality [27]. The complete lack of oncogenicity of a *Meq* knock-out mutant is in contrast to other recombinant viruses carrying deletions in other non-essential genes, as they are attenuated in virulence but still retain oncogenecity [21—26,29,30,37]. This suggests that *Meq* is the major oncogene of MDV. That said, like other oncogenic herpesviruses, the full malignant phenotypes of MDV are likely attributable to multiple viral gene products and require the collaboration of *Meq* with other MDV genes, notably vTR [21,22]. A recent paper suggests that ubiquitin-specific pro-

tease embedded in the UL36 gene of MDV also appears to play a role in transformation, perhaps in collaboration with Meq [37]. It is also noteworthy that at present we cannot completely rule out the contribution by *Meq*-vIL8, an alternate spliced form of *Meq*, and *Meq* anti-sense transcript, which are also affected by this deletion. However, previous work by Brown et al. [28] based on recombinant MDV with knock-in point mutants of *Meq* strongly suggest the critical oncogenic component is *Meq* itself.

In the present report, we evaluated the protection efficacy of rMd5ΔMeq as a vaccine after challenge with two pathotypes of virulent MDV. Our results show that rMd5ΔMeq, can fully protect chickens against lymphoma formation and death caused not only by the homologous rMd5 strain of MDV, the virus from which the rMd5ΔMeq virus was generated, but also by heterologous vv+ strain, 648A that belongs to a more virulent pathotype than rMd5.

The mechanism of vaccine protection is complex and not well understood. Upon challenged with rMd5, either rMd5ΔMeq or CVI988/Rispens vaccinated chickens had a significantly reduced viremia at all times tested (Table 4). A parallel experiment had previously determined that the virus isolated from vaccinated and challenged chickens was entirely attributable to the challenge rMd5 using monospecific antibody to the Meq protein (unpublished). We did not distinguish between the vaccine and challenge viruses in this experiment, but by analogy we conclude the isolated virus was attributable to the challenge virus. Furthermore, we have previously shown that the level of rMd5ΔMeq virus isolated from chickens was significantly lower than the wild-type rMd5 [27]. Thus, the viruses isolated at 1, 3, and 7 weeks post-challenge were entirely attributed to the challenge rMd5 virus and not rMd5ΔMeq. The ability of a vaccine virus to reduce the level of challenge virus could be a mechanism of protection by limiting the replication of challenge virus. Since the rMd5ΔMeq can become latent in infected cells, this virus could be used to study protection conferred at various time points after vaccination. Such studies will shed some light on the role of latency on protection.

In summary, disruption of the *Meq* oncoprotein not only resulted in the virus becoming attenuated, but it provided protection against very virulent plus MDV challenge in laboratory studies. These laboratory trials should be followed up with larger scale trials where vaccines are tested against early contact challenge in commercial chickens and compared to the most efficacious of the commercially available MD vaccines [15].

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