DEVELOPMENT OF A CELL LINE SYSTEM SUSCEPTIBLE TO INFECTION WITH VACCINE STRAINS OF MDV

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Summary. – Despite reliance on the need to continually prepare fresh cultures of chick embryo fibroblasts (CEFs) to make Marek's disease (MD) vaccines, MD vaccines are the most widely used vaccines in the poultry industry. Preparation of CEF's accounts for approximately 40% of the costs associated with producing MD vaccines. A significant reduction in MD vaccine production costs could be realized if a continuous cell lines were available for MD vaccine production. Recently, we reported development and characterization of a cell line system (OCL™) that supports growth and replication of oncogenic serotype 1 Marek's disease virus (MDV). Here we report development of three cell line systems for production of MD vaccine. These cell lines support the growth and replication of attenuated serotype 1 MDV (CVI-OCL™), serotype 2 MDV (SB1-OCL™) and serotype 3 MDV (HVT-OCL™). MDV is maintained in a stable state in the OCL™ cells and the infected cells can be continuously grown. The vaccines made from these cell lines are safe and protect White Leghorn chickens against challenge with very virulent serotype 1 MDV, similar to traditional vaccines made from CEF cells. These cell line systems can significantly reduce the costs associated with MD vaccine production. Furthermore, the increased stability of MDV and the potential for positive selection of recombinant MDV suggest that OCL™ will be ideal for production of more effective MDV vaccines using recombinant DNA technology.

Key words: MD vaccines; fibroblast cell lines; safety studies; pathogenesis

Introduction

MD, an economically important lymphoproliferative disease of chickens, is caused by an oncogenic, highly cell-associated avian herpesvirus, MDV (Churchill and Biggs, 1967). MDV has been divided into three serotypes (Bulow and Biggs, 1975; Lee *et al.*, 1983): serotype 1 includes all pathogenic strains and their attenuated derivatives, serotype 2 includes naturally occurring non-pathogenic isolates, and serotype 3 includes non-pathogenic but antigenically related herpesvirus of turkey (HVT) (Witter *et al.*, 1970). Since the development of live virus MD vaccines in the late 1970's, losses from MD have been significantly reduced (Calnek and Witter, 1991). The most widely used MD vaccines are live HVT or a bivalent mixture of HVT and serotype 2 MDV. The bivalent mixture synergically affords greater protection against MD, especially in those situations where HVT alone is not fully protective (Witter, 1982; Witter and Lee, 1984; Witter, 1985). More recently, several partially attenuated serotype 1 MDV vaccines, such as CVI988/Rispens and Md11/75C/R2/R23, have been introduced (Witter *et al.*, 1995).

MD vaccines are the most widely used biologics in the poultry industry. Current MD vaccines are suspensions of infected CEF cells. Since there is no sustainable cell line suitable for propagating MDV vaccine strains, the MDV vaccine industry uses primary CEF cells for production of vaccine virus (Churchill, 1985). Primary CEF cells have a finite life span (approximately three weeks) and must be prepared every week from fertile eggs from specified pathogen free (SPF) flocks, increasing the costs of producing MDV vaccines. Also, any disruption of or contamination in the supply of fertile SPF eggs will halt production of MDV vaccines.

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Many continuous cell lines that can be infected with MDV have been described (Ogura *et al.*, 1984; Lerman *et al.*, 1976; Langlois *et al.*, 1976; Akiyama *et al.*, 1973; Moscovici *et al.*, 1977; Kawaguchi *et al.*, 1987). However, these cell lines are malignantly transformed either by chemical or retroviral treatments. MDV-transformed lymphoblastoid cells are immortalized cell lines which are latently infected with MDV and usually capable of transferring MDV to CEFs *in vitro* and to susceptible chickens *in vivo* (Schat *et al.*, 1991; Akiyama and Kato, 1974; Powell *et al.*, 1974; Calnek *et al.*, 1978; Payne *et al.*, 1981; Nazarian and Witter, 1975). Since MDV is highly cell-associated and MDV vaccines consist of live infected cells, transformed cell lines are not useful for production of MDV vaccines and preclude experiments designed to study MDV oncogenicity.

Previously, we reported the development of a non-transformed fibroblastic cell line system (MDV OU2.1 and OU2.2) that supports growth and replication of serotype 1 MDV (Abujoub and Coussens, 1995, 1997). The MDV OU2 cell lines are continuous, anchorage-dependent, and carry MDV in a latent state (Abujoub and Coussens, 1997) while their morphology continues to resemble that of fibroblasts. This cell line system was originally derived from chemically immortalized CEFs (Ogura and Fujiwara, 1987). Similarly to primary CEF cells, MDV OU2.1 and OU2.2 can back transfer MDV infection to primary and secondary CEF monolayer cultures and induce MD in susceptible birds (Abujoub and Coussens, 1995). In contrast to primary CEF cells, our cell line system has unlimited life span.

We now report the development of a sustainable cell line system that supports growth and replication of vaccine strains of MDV. We present evidence that the new cell line system is safe, efficacious, and produces titers similar to MD biologics produced on traditional primary CEF cells.

Materials and Methods

Chickens from SPAFAS used in this study were SPF and negative for MDV, avian leukosis virus, reticuloendotheliosis virus, and other common poultry pathogens.

Cells and vaccine viruses. Preparation and infection of CEF cells with various strains of MDV were performed as described (Coussens and Velicer, 1988; Abujoub and Coussens, 1995). Viruses used to generate infected cell lines were serotype 3 MDV, HVT, at passage level 5. Serotype 2 MDV, SB1 strain, at passage level 10, and attenuated serotype 1 MDV, CVI/988 Rispens, were used as a virus stock to create the new cell lines. The Origen cell line (OCL™), previously known as CHCC-OU2, was maintained as described (Abujoub and Coussens, 1995). The OCL™ cells were infected with the three serotypes vaccine strains by mixing them with a highly infected stock of CEF cells. After 3 to 4 passages, cells started displaying cytopathic effect (CPE) characteristic of MDV infection. The new cell lines were called SB1-OCL™, HVT-OCL™, and CVI-OCL™, corresponding to serotypes 2, 3 and attenuated serotype 1 MDV, respectively. SB1-CEFs at passage level 29, SB1-OCL™ at passage level 5, HVT CEF at passage level 10, HVT-OCL™ at passage levels 7 and 29, and CVI-OCL™ at passage level 10 were used as vaccines. Cell-associated virus stocks consisted of a mixture of infected CEF or OCL™ cultures stored in 15% glycerol at -196°C. A bivalent vaccine composed of cell associated stocks of SB-1 and HVT in either CEF or OCL™ cultures was also used.

Pathogenic MDV strains. Stocks of virulent serotype 1 MDV strains, GA and MD11, or a very virulent strain, RB1B, growing on CEF cells were used as challenge viruses for the efficacy studies.

Safety studies were conducted in accordance with §113.300 of the 9th Code of Federal Regulations (9 CFR). Briefly, SPF chickens susceptible to MDV were divided into at least 3 separate groups (Table 1): group 1 (negative control) containing not less than 50 chickens was used as uninoculated control; group 2 (test group) (OCL™, SB1-OCL™ , HVT-OCL™ and CVI-OCL™) containing not less than 50 chickens per each test group was inoculated with 10 times as much viable virus as was contained in one dose of vaccine; group 3 (positive control) containing no less than 50 chickens was inoculated with a virulent MDV at a dosage level that was known to cause lesions of MD in at least 80% of the chickens within 50 days. For our safety study, all birds were inoculated with 100 ml of cells or virus suspension in Leibovitz's L15-McCoy 5A (LM) medium intra-abdominally. Chickens dying during the experiment were necropsied and gross lesions of MD, if any, were recorded. The sciatic plexus and any other questionable tissues were sent out for histopathological examination by the Pathology Department at Michigan State University, East Lansing, MI. The remaining birds in the positive control group were euthanized at 54 days post inoculation and all birds were examined for gross lesions of MD. The remaining groups, including the negative control group were euthanized with CO_2 at 120 days post inoculation. All birds were sexed and weighed before necropsy. Birds were then examined for gross lesions of MD and any other abnormalities.

Efficacy tests. Four separate efficacy studies were performed. In the first efficacy study, one-day-old chickens were vaccinated with 2,000 PFU and challenged on the 7th day post-vaccination with 2,000 PFU of MDV GA p8. All birds were sacrificed on the 75th day of the experiment. In the second study, HVT-OCL™ passed 7 times in OCL™ (p7) and 29 times in OCL™ (p29) were compared to HVT passed 10 times in CEF cells (p10) for ability to protect chickens against MDV. One day-old chickens were vaccinated with 2,000 PFU and challenged on the 7th day post-vaccination with 2,000 PFU of MDV GA p8. All birds were sacrificed on the 75th day of the experiment. In the third study, a bivalent vaccine consisting of 1,500 PFU of HVT-OCL™ p9 (HVT p10 passed 9 times in OCL™) and 500 PFU of SB1-OCL™ p5 (SB1 p29 passed 5 times in OCL™) was compared to a bivalent vaccine consisting of 1,500 PFU of HVT p10 and 500 PFU of SB1 p29 grown on CEF cells for ability to protect chickens against the very virulent Md11 MDV strain. One-day-old chickens were vaccinated and challenged on the 7th day post-vaccination with 2,000 PFU of Md11 MDV p17. All birds were sacrificed on the 71st day of the experiment. In the fourth study, one-day-old chickens were

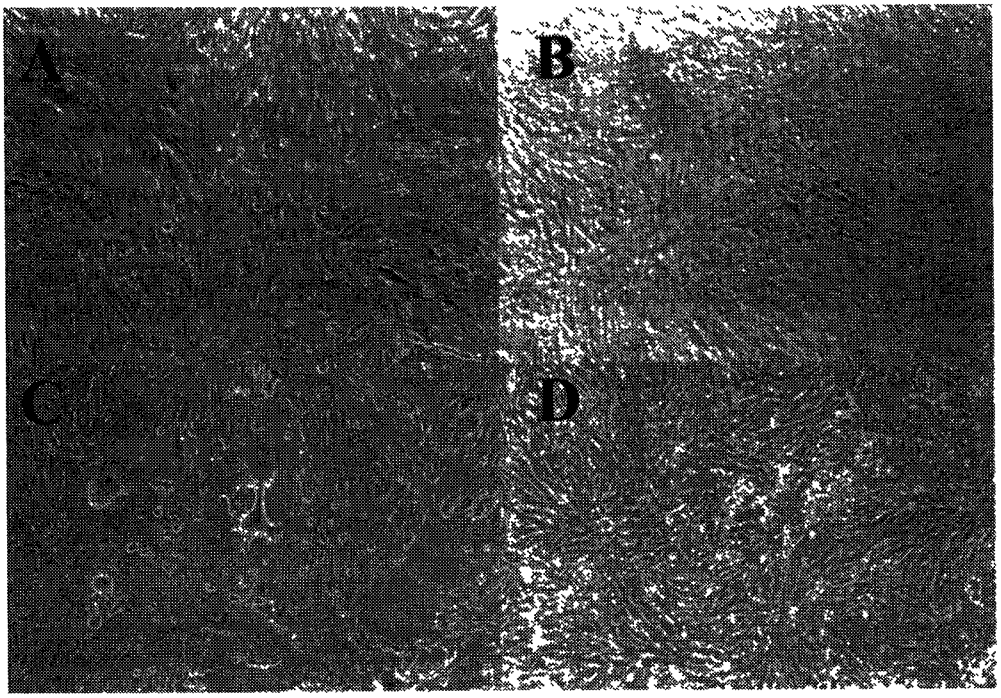

Fig. 1
Morphology of MDV-infected OCL™ cells
(A) Uninfected cells. (B) SB1-infected cells, clusters of small rounded cells characteristic of SB1 plaques. (C) HVT-infected cells, syncytia and extended regions of rounded cells. (D) CVI988-infected cells, round and spindle-shaped cells characteristic of CVI988 plaques.

vaccinated with 2,000 PFU of CVI988-OCL™ p10 and challenged on the 5th day post-vaccination with 500 PFU of RB1B MDVp2. All birds were sacrificed on the 74th day of the experiment. For all four studies, lesions and/or tumors due to MDV were determined by necropsy.

Statistical analysis. Weight data were analyzed by one-way ANOVA test of variance, for the different groups with unequal replication, in which a value of *f* of two groups was based on a variance computed from all groups and the values of *f* required for statistical significance were adjusted for the number of observations made. The percentage of MD response was calculated as the total number of MD lesions (gross and microscopic) divided by the total number of chickens in the experiment and multiplied by 100. A protective index was calculated from the percentage of MD in unvaccinated positive control minus the percentage of MD in vaccinated test group, divided by the percentage of MD in unvaccinated positive control and multiplied by 100.

Results and Discussion

Infection of OCL™ cells

The development of a sustainable cell line system that supports the growth and replication of serotype 1 MDV was reported previously (Abujoub and Coussens, 1995). This cell line, the OCL™ (Fig. 1. A) was derived by chemically mutagenizing CEFs. The cell line maintains contact inhibition and is not malignantly transformed and does not produce endogenous avian retroviruses, yet supports the growth and replication of MDV. The three viral strains (SB1, HVT and CVI/988) used in this study produced herpes-type CPE in OCL™ cultures (Fig. 1. B, C and D). Subtle difference in plaque morphology were noticed with these viruses on OCL™ and CEF cells. Serotype-2 MDV (SB1) plaques (Fig. 1. B) consisted of regions of small clusters of rounded cells loosely attached to the monolayer. Serotype 3 MDV (HVT) plaques (Fig. 1. C) consisted of big syncytia and extended regions of rounded cells. Serotype 1 MDV (CVI/988) plaques (Fig. 1. D) tended to be more polymorphic; some of the plaques were mixtures of rounded and spindle-shaped cells. Morphology of these plaques was similar to that of MDV plaques in CEF cells, but it was considerably different from what we originally reported for the oncogenic serotype 1 strain (Md11) (Abujoub and Coussens, 1995). Such variability in plaque morphology should not be surprising, particularly since different MDV strains have different biological and growth characteristics. Similarly to our previously reported cell lines, OU2.1 and OU2.2 (Abujoub

Table 1. Safety study results of OCL™ cells and OCLTT™-based vaccines

Vaccine	Inoculation dose (PFU)	Number of birds that died before 120 day	Percentage of birds that survived 120 day	Gross MD/total	Average body weights[b]
Uninoculated negative control	–	1	98	0/50	1840.0
OCL™	1 x 10^6 cells	1	98	0/50	1884.0
SB1-OCL™	40,000	6	88	0/50	1816.0
HVT-OCL™	100,000	5	90	0/50	1800.0
CVI-OCL™	40,000	8	84	0/50	1816.0
GA/CEF	2,800	50[a]	0	42/50	ND

[a]Positive control chickens were euthanized 50 days post inoculation.
[b]Corrected body weights; weights of femaleswere multiplied by the ratio of average weights of males to females within each group to correct for weight differences due to different sex.
ND = not determined.

and Coussens, 1997), all new infected cell lines maintained virus in a latent state when sub-confluent, produced infectious virus when confluent, and could transfer virus to CEF cells and chickens (data not shown).

Safety study

Previously, we successfully demonstrated that uninfected OCL™ cells can be administrated into chickens without any evidence of tumor formation or illness (Abujoub and Coussens, 1997). However, in order to use the OCL™ cells for production of MD vaccines, we needed show that both the cells and virus in the cells were safe in chickens as outlined in the U.S. Department of Agriculture Safety Test for Master Seed Stocks of MDV vaccines, 9 CFR §113.330. Upon completion of the safety study, no gross lesions were observed in any of the chickens injected with 10^6 of uninfected OCL™ cells or with 10 times the normal vaccine dose of SB1-OCL™, HVT-OCL™ and CVI-OCL™ cells (Table 1). The GA strain, used as a positive control induced gross lesion of MD in (42/50) 84% of inoculated chickens (Table 1). One way ANOVA analysis of the variance with unequal treatments in each group was used to examine a null hypothesis, which stated that there was no statistically significant difference among the average body weights in the 4 test groups (OCL™, CVI-OCL™, HVT-OCL™ and SB1-OCL™) and the uninoculated negative control group of chickens. Using StatMost for Windows, we clearly demonstrated that F_{cal} (1.96) < F_{tab} (2.66). Therefore we accepted the null hypothesis. The ANOVA results and the average body weights (Table 1) presented in Fig. 2 strongly support a conclusion that the safety study has met the criteria required by the 9 CFR. The data presented in this and previous studies (Abujoub and Coussens, 1997) demonstrated that these cells and viruses can be administrated safely to chickens and are a suitable alternative to MD biologics prepared on CEF cells.

Efficacy studies

We conducted a series of efficacy studies that demonstrated that MD vaccines made with OCL™ cells were as effective as MD vaccines made with CEF cells. The results of an efficacy study comparing HVT-OCL™ passed 7 times in OCL™ (p7) compared to HVT passed 10 times in CEF cells (p10) for the ability to protect chickens against the MDV GA strain are in Table 2 which shows that, when compared to HVT produced by conventional methods on CEF cells, HVT-OCL™ was fully protective against GA. The results of an efficacy study that compared HVT-OCL™ passed 7 times in OCL™ (p7) and 29 times in OCL™ (p29) to HVT at passage 10 on CEF are shown in Table 3 which shows that HVT-OCL™ at either passage level was as efficacious in ability to protect chickens against MDV as HVT passed 10 times in CEF cells (p10). The results of the efficacy study are summarized in Table 4. Comparison of a bivalent vaccine consisting of HVT-OCL™ p9 (HVT p10

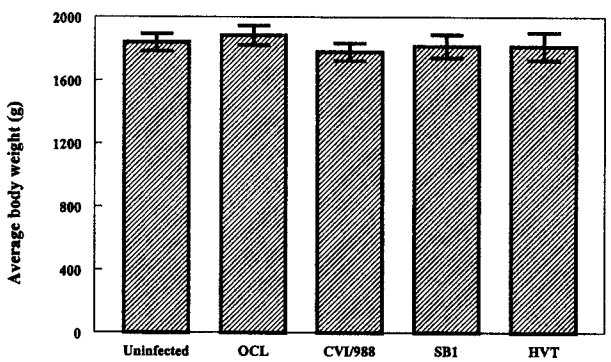

Fig. 2
Comparison of average body weights of test and negative control groups

Table 2. Comparison of HVT-OCL™ vaccines to conventional HVT vaccines

Vaccine	Titer (PFU/ml)	No. of protected/total birds	Protection (%)
HVT-OCL™ p7	2000	13/13	100
HVT-CEF p10	2000	13/13	100
Challenge control	–	2/10	–
Negative control	–	12/12	–

Table 3. Comparison of highly passaged HVT-OCL™ to low passaged HVT-OCLTM™ and conventional HVT vaccines

Vaccine	Titer (PFU/ml)	No. of protected/total birds	Protection (%)
HVT-OCL™ p7	2000	26/30	87
HVT-OCL™ p29	2000	27/29	93
HVT-CEF p10	2000	27/30	90
Challenge control	–	0/15	–

Table 4. Comparison of HVT-OCL™/SB1-OCL™ bivalent vaccine to conventional bivalent vaccines

Vaccine	Titer (PFU/ml)	No. of protected/total birds	Protection (%)
HVT-OCL™ p9	1,500	13/15	87
SB1-OCL™ p5	500		
HVT-CEF p10	1,500	15/15	100
SB1-CEF p29	500		
Challenge control	–	0/10	–
Negative control	–	10/10	–

Table 5. Protection by CVI988-OCL™ vaccine against very virulent MDV RB1B

Vaccine	Titer (PFU/ml)	No. of protected/total birds	Protection (%)
CVI988-OCL™ p7	2000	46/50	92
Challenge control	–	0/19	–

passed 9 times in OCL™) and SB1-OCL™ p5 (SB1 p29 passed 5 times in OCL™) with a bivalent vaccine consisting of HVT p10 and SB1 p29 grown on CEFs also showed no difference in the level of protection against challenge with the very virulent Md11 MDV strain. Finally, the ability of CVI988-OCL™ passed 10 times in OCL™ to protect chickens against the very virulent RB1B MDV strain is shown in Table 5 which demonstrates that the vaccine is efficacious against RB1B.

All of these results show that there is no detectable difference in efficacy between vaccines prepared from OCL™ cells an those prepared from CEF cells.

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