

*Historical Article*—**History of the First-Generation Marek's Disease Vaccines: The Science and Little-Known Facts**Karel A. Schat<sup>AB</sup><sup>A</sup>Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853

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**SUMMARY.** Shortly after the isolation of Marek's disease (MD) herpesvirus (MDV) in the late 1960s vaccines were developed in England, the United States, and The Netherlands. Biggs and associates at the Houghton Poultry Research Station (HPRS) in England attenuated HPRS-16, the first cell-culture-isolated MDV strain, by passaging HPRS-16 in chick kidney cells. Although HPRS-16/Att was the first commercially available vaccine, it never became widely used and was soon replaced by the FC126 strain of herpesvirus of turkeys (HVT) vaccine developed by Witter and associates at the Regional Poultry Research Laboratory (now Avian Disease and Oncology Laboratory [ADOL]) in East Lansing, MI. Ironically, Kawamura *et al.* isolated a herpesvirus from kidney cell cultures from turkeys in 1969 but never realized its potential as a vaccine against MD. Rispens of the Central Veterinary Institute (CVI) developed the third vaccine. His associate, Maas, had found commercial flocks of chickens with MDV antibodies but without MD. Subsequently, Rispens isolated a very low pathogenic strain from hen number 988 from his MD antibody-positive flock, which was free of avian leukosis virus and clinical MD. This isolate became the CVI-988 vaccine used mostly in The Netherlands. During the late 1970s, HVT was no longer fully protective against some new emerging field strains. The addition of SB-1, isolated by Schat and Calnek, to HVT improved protection against the emerging very virulent strains. In the 1990s CVI-988 became the worldwide vaccine gold standard. This review will present data from published papers and personal communications providing additional information about the exciting 15-yr period after the isolation of MDV to the development of the different vaccines.

**RESUMEN.** *Artículo Histórico* - Historia de las vacunas de primera generación contra la enfermedad de Marek: La ciencia y algunos datos poco conocidos.

Poco después del aislamiento del herpesvirus de la enfermedad de Marek (MDV) a finales de la década de los 1960s se desarrollaron vacunas en Inglaterra, en los Estados Unidos y en los Países Bajos. Biggs y sus colaboradores en la Estación de Investigación Avícola en Houghton (con las siglas en inglés HPRS) en Inglaterra, atenuaron el virus HPRS-16, que fue la primera cepa del virus de Marek aislada en cultivo celular y fue atenuada mediante pasajes en cultivos celulares de células de riñón de pollo. Aunque la cepa HPRS-16/Att fue la primera vacuna disponible comercialmente, nunca llegó a ser ampliamente utilizada y pronto fue sustituida por la cepa FC126 que es una vacuna con un herpesvirus de pavos (HVT) desarrollada por Witter y colaboradores en el Laboratorio de Investigación Avícola Regional (ahora llamado Laboratorio de Enfermedades y Oncología Aviarias [ADOL]) en East Lansing, MI. Irónicamente, Kawamura *et al.* aislaron un virus herpes a partir de cultivos celulares de riñón de pavos en 1969 pero nunca se dieron cuenta de su potencial como vacuna contra la enfermedad de Marek. Rispens del Instituto Veterinario Central (CVI) desarrolló una tercera vacuna. Su colaborador, Maas, había encontrado parvadas comerciales de pollos con anticuerpos contra el virus de Marek pero sin la enfermedad. Posteriormente, Rispens aisló una cepa de muy baja patogenicidad patógena de la gallina número 988 de su parvada positiva a la presencia de anticuerpos contra la enfermedad de Marek, que estaba libre del virus de la leucosis aviar y de la enfermedad de Marek clínica. Este aislamiento se convirtió en la vacuna CVI-988 utilizada sobre todo en los Países Bajos. Durante la década de los 1970s, la vacuna HVT ya no era totalmente protectora contra algunas nuevas cepas de campo emergentes. La adición de la vacuna SB-1, aislada por Schat y Calnek, a la vacuna HVT mejoró la protección contra las cepas emergentes muy virulentas. En la década de los 1990s la vacuna CVI-988 se convirtió en el estándar de oro de vacunas en todo el mundo. Esta revisión presenta datos de los artículos publicados y comunicaciones personales que proporcionan información adicional acerca del apasionante periodo de 15 años después del aislamiento del virus de la enfermedad de Marek hasta el desarrollo de las diferentes vacunas.

**Key words:** CVI-988, FC126, herpesvirus of turkeys, Marek's disease, Marek's disease virus, SB-1, vaccines

**Abbreviations:** AAAP = American Association of Avian Pathologists; ADOL = Avian Disease and Oncology Laboratory; ALV = avian leukosis virus; CEF = chicken embryo fibroblasts; CKC = chick kidney cells; CPE = cytopathic effect; CVI = Central Veterinary Institute; DEF = duck embryo fibroblasts; HPRS = Houghton Poultry Research Station; HVT = herpes virus of turkeys; LAH = Lohman Animal Health; LEU = Leukosis Experimental Unit; MD = Marek's disease; MDV = Marek's disease virus; p = passage; PBL = peripheral blood lymphocytes; PDRC = Poultry Disease Research Center; RPRL = Regional Poultry Research Laboratory; SPF = specific-pathogen-free; THV = turkey hepatitis virus, turkey herpesvirus; TNO = Organization for Applied Scientific Research in The Netherlands; UGA = University of Georgia; USDA = U.S. Department of Agriculture; vv = very virulent; WRL = Wellcome Research Laboratories; WHG = Wisconsin-Harvard-Georgia; WTHV = Wisconsin turkey herpesvirus

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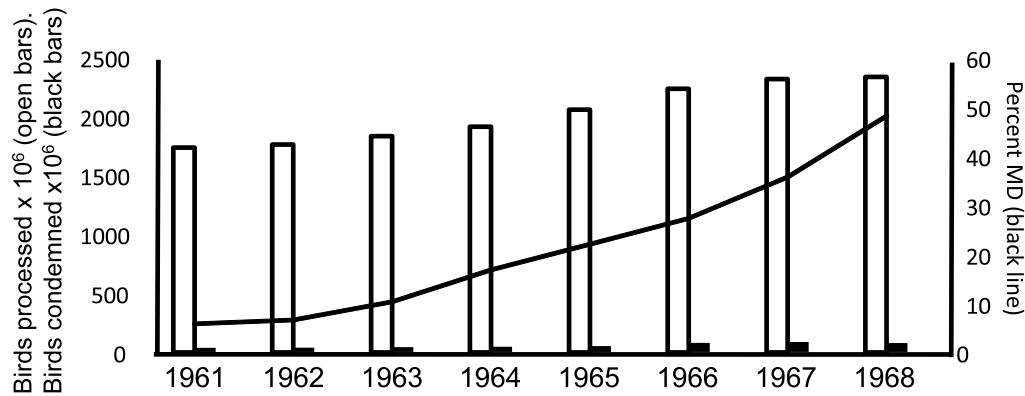


Fig. 1. Total number of birds processed (open bars) and total number of birds condemned (closed bars) between 1961 and 1968. The percentage of the birds condemned for leukosis (MD = Marek's disease) shows a rapid increase from approximately 5% in 1961 to 50% in 1968 (black line). Data adapted from Reference 14.

## INTRODUCTION

Marek's disease (MD), a herpesvirus-induced tumor disease of chickens, is extremely well controlled by vaccination (reviewed in, e.g., 3,27,34). These authors, however, express concerns about the potential of vaccine-driven increases in pathogenicity, although there is little evidence that a further increase in virulence has occurred since Witter (61) described the very virulent (vv)+ strains of Marek's disease virus (MDV). Discussions during the 10th International Symposium on Marek's Disease and Avian Herpesviruses in 2014 and the symposium "An Update on Marek's Disease Vaccination, Diagnosis, and Immunosuppression" organized by the American Association of Avian Pathologists (AAAP) in 2015 indicated little concern that the MD situation was changing. Breaks and leukosis (the U.S. Department of Agriculture [USDA] classification for MD) condemnations still do occur in the broiler industry, but only at very low levels compared to the situation in the 1960s. From 1961 to 1968 there was a slight increase in the total number of condemnations (Fig. 1). However, there was a marked change in the percentage of birds with leukosis. In 1961 about 5% of the total condemnations were attributed to MD, which increased to 50% of the total condemnations in 1968 (Fig. 1) (14). Likewise, the situation in the layer industry was desperate in the 1960s with up to 50% MD mortalities (30). Thus, when MDV was isolated in cell culture in 1967 and 1968 by two different groups (24,67) the race was on to develop vaccines against MD. Within 4 yr three different vaccines were available (26,42,49) (Table 1). In this article important events leading to the development of monovalent and bivalent vaccines will be reviewed with an emphasis on behind-the-scenes information rather than the science of MD vaccines and vaccine-induced protection. The latter has been extensively reviewed

(e.g., 3,12,62). The following sources were used extensively for this paper: historical articles in *Avian Diseases* (13,35), biographies in the AAAP archives (4,16,36,43,63,64,65), the CD describing the historical programs presented at the 5th and 10th International Symposia on Marek's disease in East Lansing, MI (30), and personal communications from many of the key researchers of that time.

## HPRS-16/ATT: THE FIRST VACCINE

Professor Peter Martin Biggs (Supplemental Fig. S1A,B) joined the Houghton Poultry Research Station (HPRS), England (Supplemental Fig. S2A,B) in 1959 to work specifically on the avian leukosis complex when the U.K. Agricultural Research Council decided to fund a program on this complex disease. A special facility, the Leukosis Experimental Unit (LEU) (Supplemental Fig. S3) was designed by him for this project and opened in January 1962 (4). Dr. Laurence N. (Jim) Payne (Supplemental Fig. S1C) joined the LEU in 1961 and remained at the HPRS until 1992 when HPRS was closed, and then was at the Institute for Animal Health in Compton until retirement in 1997 (43). Payne had previously worked with Biggs on avian viral tumors at the Field Station of the Bristol Veterinary School in Langford. To complete the original MD research team, Biggs hired Roger Chubb in 1963 and Tony Churchill (Supplemental Fig. S4) in 1966. Tony did his Ph.D. thesis research on MD while working at HPRS (4). One of the first breakthroughs was the successful *in vivo* transmission of MD with the use of a crude tumor suspension of the B14 strain with subsequent transmissions with peripheral blood lymphocytes (PBL) (6). The MD strain HPRS-16 also was first isolated by *in vivo* transmission experiments using PBL or tumor homogenates from

Table 1. Overview of vaccine strains developed between 1969 and 1978.

Vaccine	Serotype	Year published (Reference)	Currently used	U.S. patent number	Date of filing	Date of publication
HPRS-16/Att	1	1969 (26)	No	3674861 <sup>A</sup>	October 31, 1969	July 4, 1972
HVT FC126	3	1970 (42)	Yes	3642574	April 29, 1970	February 15, 1972
CVI988	1	1972 (49)	Yes	Not patented	NA	NA
SB-1	2	1978 (51)	Yes <sup>B</sup>	4160024	May 1, 1978	July 3, 1979

<sup>A</sup>A UK patent was filed, and the commercialization was handed over to the National Research Development Corporation (Biggs, personal communication 2014).

<sup>B</sup>Used only as part of bivalent or trivalent vaccines.

Table 2. Results of the first four field trials using HPRS-16/Att. Adapted from Biggs *et al.* (8).

Trial	Vaccine	Age at vaccination in days	No. chickens	Percent mortality <sup>A</sup>		
				Total	MD	NSD
1	–	–	2,443	12.9	1.8	11.1
	+	1	1,078	5.5 <sup>B</sup>	0.2 <sup>B</sup>	5.3 <sup>B</sup>
2	+	21	1,207	6.1 <sup>B</sup>	0.2 <sup>B</sup>	5.9 <sup>B</sup>
	–	–	1,952	4.5	–	–
	+	1	886	6.2	–	–
3	+	21	978	4.7	–	–
	–	–	5,855	42.5	22.9	19.6
4	+	21	825	16.4 <sup>B</sup>	4.5 <sup>B</sup>	11.9 <sup>B</sup>
	–	–	2,583	69.4	–	–
	+	21	877	17.9 <sup>B</sup>	–	–

<sup>A</sup>Total mortality is based on 9 to 57–60 wk of age, – = no breakdown in MD and NSD (nonspecific disease) available at the termination of the experiment.

<sup>B</sup>Significantly different from unvaccinated controls.

chickens with the so-called “acute leukosis” (9). The finding that whole cells were essential for the transmission of MD (7) was the key to the subsequent isolation in chick kidney cell (CKC) cultures of HPRS-16 by Churchill and Biggs (24). HPRS-16 was also strictly cell-associated in cell culture, which at the time was believed to be a problem for the production of a vaccine. It was hoped that cell-free HPRS-16 could be obtained by making passages in CKC at appropriate intervals. However, the virus remained strictly cell-associated when tested between passages (p) 33 and 80 (23,25). When Payne used p31 in two pathogenesis experiments, it was, to his surprise, no longer pathogenic (Payne, personal communication, 2014; Biggs, personal communication, 2014). These birds were then used for challenge experiments with virulent p14 HPRS-16 and were shown to be protected (Payne, personal communication, 2014). These data and the results of two additional experiments in which chickens were vaccinated by intra-abdominal injection at 1 or 14 days of age with HPRS-16 p39 and challenged with low passage HPRS-16 at 4 wk of age were reported in the first paper describing successful immunization against MD (26). The attenuated virus had lost the so-called A antigen (now known to be glycoprotein C) (25) and was no longer able to spread horizontally (26). Because MDV is strictly cell-associated, the cells containing the vaccine virus needed to be frozen in a controlled way and stored in liquid nitrogen, which was novel at the time for any vaccine. The use of all MDV vaccine strains as cell-associated vaccines requiring storage in liquid nitrogen is still common practice. Field trials were initiated in 1968 by the Houghton team together with David Powell (veterinarian for Cobb Breeding Company) and Tony Harris (veterinarian for Ross Poultry Ltd.). Biggs (personal communication, 2014) mentioned the following:

These trials are particularly memorable for a number of reasons. Firstly, we learned how important it was to seal the ampoules fully before freezing as one, which clearly had not been properly sealed, exploded when put into water to thaw. Luckily I had already withdrawn my head from overlooking the process. A narrow escape! Second, in one of the trials we used gentian violet to mark vaccinated chicks to distinguish them from unvaccinated chicks in the same pen. This job was delegated to Tony Harris who started out using surgical gloves but found

they quickly became punctured and so disbanded using them. At the end he found the gentian violet would not wash off his hands which had become well and truly stained. This lasted over a week much to his embarrassment. He found it impossible to hide it from others thus stimulating a range of questions.

Interestingly, vaccination was done by intra-abdominal inoculation at 1 day of age (Experiments 1 and 2) or at 21 days of age (Experiments 1–4) with p45 to p48 of HPRS-16/Att. MD challenge was low in Experiments 1 and 2 and moderate in Experiments 3 and 4 (Table 2) (8). In hindsight the low challenge was lucky, because early challenge would most likely have caused considerable mortality. In a subsequent field experiment HPRS-16/att chickens vaccinated at 1 day of age had 15.2% MD at 60 wk of age, whereas nonvaccinated birds had 51.1% MD (5).

Around the same time W. (Bill) Baxendale and his team at Wellcome Research Laboratories (WRL) had attenuated the Beckenham isolate, which was claimed to be naturally avirulent. The attenuated virus no longer spread horizontally and no longer produced the A antigen. It is not clear if this virus is similar to or more virulent than the CVI-988 strain of Dr. Rispens. Field trials with the attenuated Beckenham strain (Marivax<sup>®</sup>) were conducted by Blaxland *et al.* (10) and Meulemans *et al.* (41). The results showed protection but the MD mortality in nonvaccinated birds was in general rather low. Baxendale (Supplemental Fig. S5) left WRL in 1969 or 1970 to join Tony Churchill's company Poultry Biologics Ltd. Churchill together with Cliff Wannop (Department of Clinical Pathology of HPRS) had decided to leave HPRS around 1968/1969 to start the vaccine company Poultry Biologics Ltd. in 1969 (Biggs, personal communication, 2014; Churchill, personal communication, 2016). The company bought an old estate, The Elms (Supplemental Fig. S6) in Houghton, roughly 1 mile from HPRS. HPRS-16/Att was produced for a few years until herpes virus of turkeys (HVT) became the preferred vaccine. Poultry Biologics Ltd. was sold to Intervet (now MSD) in 1972, which kept The Elms for research for a long time with Baxendale as one of the chief scientists.

Scott McLeod and Tony Harris used the first commercial MD vaccine batch produced by Poultry Biologics (McLeod, personal communication, 2016). McLeod made the following comment:

Tony and I used the very first commercial batch of MDV vaccine on our pedigree chickens at some point in 1970. I had to drive down from Edinburgh to Churchill's vaccine facility near St. Ives (about 300 miles) with a large liquid nitrogen container (10 liters of fluid) on the passenger seat of my car, pick up several hundred glass vials of MDV vaccine and make the return journey to Edinburgh, just as well there were no Health and Safety regulations then. The protocol was to transfer a few glass vials from the liquid nitrogen directly into a plastic bowl containing warm water at 37 C, I would estimate that 20–30% of the vials exploded during the thawing process.

Sealing ampoules was not only a problem at HPRS but also at Poultry Biologics, certainly in the beginning!

Although HPRS-16/att was soon eclipsed by HVT as the vaccine of choice in most of the world, its use was the first demonstration that vaccination against MD was possible. As such it was one of the great contributions to Marek's disease research in general.

### HVT BECAME THE MAIN VACCINE IN THE 1970s

Two research groups were involved in the original work isolating HVT. The team of Dr. David (Dave) P. Anderson at the University of Wisconsin was the first to isolate a herpesvirus from turkeys but they did not realize the potential usefulness. Anderson established a flock of turkeys from progeny of mycoplasma-free turkeys established at Iowa State University by Dr. M. S. Hofstad and Dr. H. W. Yoder, Jr. (1). These turkeys were used by Dr. Hitoshi Kawamura to establish turkey kidney cell cultures in attempts to isolate reovirus (37). However, on seven occasions syncytia with intranuclear inclusions were observed. In the summer of 1968 one of the syncytial plaques was selected for further passage in turkey kidney cell cultures lacking these cytopathic effects (CPE). Based on a number of assays the agent was classified as a cell-associated herpesvirus designated as Wisconsin turkey herpesvirus (WTHV-1) (38). The virus was submitted to the turkey virus repository at the Harvard Primate Center after two passages in turkey cells (31), where it was confirmed to be a herpesvirus (1). WTHV-1 was lyophilized and put in the freezer, because the laboratory of Anderson was working on identifying the cause of bluecomb disease. At that time reovirus was one of the candidate pathogens for bluecomb, which is now known as turkey coronavirus enteritis. Shortly afterwards, Kawamura returned to Japan and Anderson left the University of Wisconsin to accept the position of chairman of the Poultry Diagnostic and Research Center (PDRC) at the University of Georgia (UGA).

Concerns about devastating mortality from avian leukosis and range paralysis (the neural form of MD) in growing and mature chickens led in 1938 to the creation by the USDA of the Regional Poultry Research Laboratory (RPRL) in East Lansing, MI, now Avian Disease and Oncology Laboratory (ADOL) (Supplemental Fig. S7). The detailed history of ADOL and its important role in research on avian tumor viruses has recently been reviewed by Witter (66). Both names, RPRL and ADOL, are used in this paper.

Around the time that Kawamura isolated WTHV-1, Dr. Richard L. (Dick) Witter (Fig. 2), working since 1964 at ADOL, started a project on isolating MDV from broiler flocks and also from turkey flocks, which occasionally experienced lymphoid tumors. The following information was provided by Dr. Witter (personal communication, 2016; 64). On September 24, 1968 Witter received 10 turkeys from a 23-wk-old flock on the Ernie Schrock farm (New Paris, IN) through Dr. Joe Ostendorf, DVM, located in Milford, IN. The turkeys were entered in a single book, which was used at that time for submissions of field cases. The turkeys were entered as Field Case 126, hence the designation FC126 for the virus. Eight of the 10 turkeys were positive for virus in duck embryo fibroblasts (DEF) and CKC. Interestingly, during his sabbatical year (1966–1967) at RPRL Dr. Bart Rispens convinced Dr. Graham Purchase to bring in DEF for his work on nonproducer cells (44). Dr. John Solomon, working with Witter, used the DEF for the isolation of FC126 because DEF cells grew much better than chicken embryo fibroblasts (CEF) (Witter, personal communication, 2016). Isolates from Turkeys 4 and 6 were pooled at the sixth passage in DEF, which was then identified as FC126 and used for the original experiments at p11 or less. Cloned virus was prepared with the use of cell-free FC126 at p13. Individual plaques were selected and used for research starting at p16. In 1985 Witter deposited FC126 with the American Type Culture Collection (catalogue VR-584B), from which it is available upon request. Witter and co-workers found that

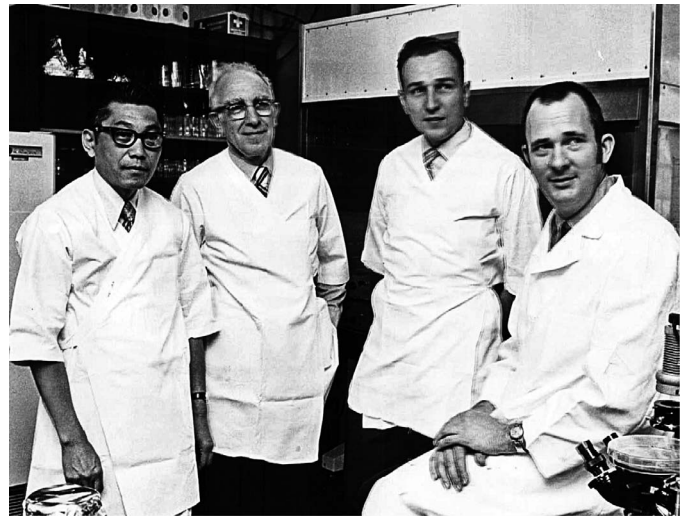


Fig. 2. From left to right: Drs. W. (Bill) Okazaki, B. R. Burmester, H. G. (Graham) Purchase, and R. L. (Dick) Witter circa 1970 at the Regional Poultry Research Laboratory (now Avian Disease and Oncology Laboratory) in East Lansing, MI. These investigators played key roles in the isolation and development of the HVT FC126 vaccine. The photo, courtesy of Dick Witter, was published in Reference 66.

HVT and MDV shared some antigens based on serology, including indirect immunofluorescent tests. Moreover the virus was non-pathogenic in chickens and turkeys (71).

In November 1968, Witter met Anderson at the American Cyanamid 10th Poultry Pathologists Conference in Princeton, NJ, where Witter learned that the Anderson team had isolated herpesvirus from healthy adult turkeys. Witter indicated that they had also isolated several herpesviruses from turkeys. Anderson sent the WTHV-1 isolate to Witter without a Memorandum of Understanding, something that today would be impossible. Witter confirmed that FC126 and WTHV-1 were similar viruses.

Witter presented his findings on June 23, 1969 at the 41st Northeastern Conference on Avian Diseases in Orono, ME. He identified the virus as turkey herpesvirus (THV). Dr. Bruce Calnek (personal communication, 2016) immediately stood up and told Witter that the acronym THV stood for turkey hepatitis virus described by Snoeyenbos *et al.* in 1959 (54). After returning to East Lansing Witter then changed the name from THV to herpesvirus of turkeys (HVT).

After the report by the Houghton team that attenuated MDV could protect chickens against MD many groups initiated similar experiments. At ADOL, Dr. W. Okazaki had started attenuation of their MDV isolates with the aim to develop a vaccine. Witter offered to include his FC126 virus in protection studies (64), which resulted in the paper showing that HVT protected very well against challenge with the JM strain of MDV (42). Interestingly, this paper never mentions any results of protection studies using attenuated MDV. Another interesting observation is the absence of Witter as co-author on the paper by Okazaki *et al.* (42). Witter commented on this in his biography as follows:

Peter Biggs asked me once why I gave up FC126 for someone else to develop as a vaccine. In retrospect, however, I gained much from my role in the development of the FC126 vaccine, even though my name did not appear on the seminal paper (64).

Dr. C. S. (Cas) Eidson was attenuating the GA strain of MDV when Dave Anderson joined PDRC at UGA on July 1, 1969 (M. Jackwood, personal communication, 2016). Eidson had basically followed the early work by Biggs and Payne (7) passing tumor cells at 4-wk intervals starting with cells from an ovarian tumor obtained from a 9-wk-old bird. Anderson retrieved WTHV-1 from the Harvard Primate Center, where it had been passed 10 times in a chick heart cell line and was now designated the Wisconsin-Harvard-Georgia (WHG) isolate (31). Initial MD protection experiments were conducted with the use of GA p42 to 88 and WHG p7 to 53. I assume that the passage levels for WHG refer to passages made at PDRC. Both types of vaccines provided satisfactory results (31). With permission of the State Veterinarian of Georgia 17 field trials were conducted in North Georgia involving close to 400,000 birds (1,32). Cell-associated HVT strains WHG and FC126 offered good protection against MD morbidity and mortality, while attenuated GA provided only partial protection. Interestingly, the vaccines were prepared as freshly trypsinized cultures and used within 2 to 3 hr after harvest.

While the vaccine experiments were in progress the industry was still suffering and applying approaches to control MD that were actually outright dangerous. Dr. Walter Hughes (36) working for Kimber Farms mentioned the addition of litter from houses with low levels of MD to the litter on which newborn chicks were brooded. After the news became known that turkey blood was commonly positive for a herpesvirus that could protect against MD, turkey blood was used to vaccinate. Hughes stated (36)

In the summer of 1969 Kimber Farms Turkey Breeding Division transferred a group of adult Toms to our Laboratory isolation. Inoculation with 0.2 ml of pooled fresh blood was made available for chicks destined for chronic high loss farms. This procedure was effective in reducing loss. It was, however, quite costly at eight cents per chick.

It is most likely that the practice of injecting turkey blood was used elsewhere in the United States as well, probably resulting in the introduction of turkey pathogens into chickens. Kimber Farms obtained FC126 from Witter in 1969 and developed a vaccine production unit. The test results on 450,000 chickens were submitted in a California license application, which was granted in 1971 as the first state-level license in the United States. Kimber Farms continued their own HVT vaccine production until 1974 when the Kimber Farms closed. The claim by Hughes (36) that this was the first state license for the HVT vaccine may not be correct. Burmester and Purchase (13) mentioned that the State of Michigan licensed the vaccine in November 1970. At least one private company operated by Lee Noll, a former technician working with Drs. Purchase and Okazaki, sold the vaccine under state license for a short time (Witter, personal communication, 2016). Based on the work by Eidson and associates the vaccine was also licensed by the State of Georgia in 1970. The USDA licensed the vaccine in March 1971.

When RPRL announced in June 1969 that HVT protects chickens against MD, the industry immediately wanted to obtain the seed virus to initiate the development of commercial vaccines. Witter (personal communication, 2016) mentioned that Dr. Okazaki distributed FC126 to a number of companies and laboratories probably in 1969–1970, but this list is not available. On July 29 1969, Dr. Salsbury Laboratories, Merck and Sterwin Laboratories were the first vaccine companies receiving seed material

from RPRL (35,63). Dr. Hiram Lasher was at that time president of Sterwin Laboratories, Inc., located in Millsboro, DE. He immediately started field trials together with the University of Delaware using FC126 produced in CEF. Results were excellent and he needed the USDA license in a hurry, which was granted on March 1, 1971 (63). A newspaper clipping (source unknown) dated March 6, 1971 stated that the three companies announced the start of selling the vaccine as early as April 1. Dr. Salsbury Laboratories also produced the vaccine in CEF derived from specific-pathogen-free (SPF) chickens, whereas Merck used DEF. They had developed a SPF Khaki Campbell duck flock, which was sold to SPAFAS when Merck decided to stop the production of the MD vaccine. Ultimately, the SPF duck flock ended up for a time at Cornell University to support the research at the International Duck Research Cooperative on Long Island, NY (15).

There is some information on when FC126 was produced in Europe. Dr. Egon Vielitz (personal communication, 2016), working for Lohman Animal Health (LAH) in Germany, received FC126 from Witter when he visited East Lansing in 1970. In 1971 LAH started to vaccinate large flocks in Spain. This is interesting because the newspaper mentioned before, dated March 6, 1971, included an article stating that Spain banned the import of chickens vaccinated with FC126! Dr. Daniel Gaudry (personal communication, 2016), working for Mérieux in France, visited RPRL in the fall of 1970 and returned to France with FC126. It is less clear when FC126 was first produced in the Netherlands. CVI-988 was, and still is, the preferred vaccine in The Netherlands, but as early as 1977 the combined vaccine CVI-988 + FC126 was used on selected farms (J.C. van den Wijngaard, personal communication, 2016) and was likely produced by Laboratory Dr. de Zeeuw (part of Gist-Brocades Animal Health). Dr. R. Hein (personal communication, 2016) mentioned that Intervet (now MSD) started producing FC126 in The Netherlands after acquiring Laboratory Dr. de Zeeuw in 1988.

One of the problems with both the attenuated MDV as well as HVT was the cell-associated nature of both viruses. It was hoped that cost-effective cell-free vaccines could be developed, especially after Calnek *et al.* (18) published a method to develop cell-free HVT. Indeed cell-free HVT vaccines were produced by several companies as early as 1973–1974. (e.g., American Scientific Laboratories, see 33). Early reports suggested that cell-free HVT was equally protective as cell-associated HVT even in the presence of maternal antibodies against HVT (33,76). However, cell-free HVT never became fully accepted in commercial poultry settings, probably because cell-free HVT is more susceptible to maternal antibodies than cell-associated vaccines (68). Cell-free HVT is still produced, but mostly for use in small backyard flocks or places where the availability of liquid nitrogen is problematic.

#### **HOW CVI-988, ALSO KNOWN AS RISPENS' VACCINE, CONQUERED THE WORLD AFTER BEING USED IN THE NETHERLANDS**

Similar to the MD problems in the United States and the United Kingdom, The Netherlands experienced extremely high MD mortality especially in layer and breeder flocks. In 1964, under pressure from poultry producers, the “Productschap voor Pluimvee en Eieren” (Production Board for Poultry and Eggs, which existed from 1956 to January 1, 2015) approached the Dutch Organization for Applied Scientific Research (TNO) and the Central Veterinary Institute (CVI) to initiate a project to study the avian leukosis

Table 3. Field trials comparing HPRS-16/Att and CVI-988 in 1970.<sup>A</sup>

Vaccine	Age in weeks when examined	Vaccinated		Controls	
		No. of birds	% mortality	No. of birds	% mortality
HPRS-16/Att	11–19	13,750	3.4	13,600	4.4
	11–21	3000	17.8	2300	20.2
	11–22	2000	9.5	3800	16.8
CVI-988	11–17	3000	3.2	19,100	10.9
	11–19	8600	0.9	23,900	9.4
	11–21	2500	3.0	5000	11.9

<sup>A</sup>Data provided by Dr. M. Krasselt (Laboratory Dr. de Zeeuw, Gist-Brocades) to Dr. Rispens and published in *Tecnica Pecuaria en Mexico* in 1971 (47).

complex. Shortly afterwards and with considerable financial support from the Dutch government the avian leukosis study group was started with veterinary pathologist Henk J. L. Maas and Dr. Bart Helmich Rispens (Supplemental Fig. S9) as investigators for the TNO and CVI teams, respectively. The TNO group was originally housed at the Poultry Health Institute first in Soesterberg and later in Doorn. At the start of the project, the CVI team was located in Rotterdam (Supplemental Fig. S8A), where a small building was used for virus research (Supplemental Fig. S8B). In 1971, both groups moved to new state-of-the-art facilities in Lelystad. According to the (bi)annual reports (2) of the avian leukosis study group, the first 3 yr were mostly devoted to the development of the necessary infrastructure and research on avian leukosis virus (ALV) – associated aspects of the avian leukosis complex. The switch to very intensive research on MD occurred in 1967 when Rispens returned from his sabbatical year at RPRL in East Lansing, MI. A cell-culture system was established for the isolation of MDV with DEF. These cells were selected because ducks are free of ALV. Maas (personal communication, 2013; 11,46,48,49) started to collect many samples from flocks with MD problems, which were positive for virus isolation in DEF and antibodies in an indirect immunofluorescence assay. Interestingly, samples from flocks without history of clinical MD were also positive in both tests. Inoculation of 1-day-old chickens with isolates from the latter flocks showed that large differences in MD pathogenicity existed (49). Preliminary challenge experiments of chickens inoculated with a low-pathogenicity isolate showed protection (46,48). Rispens decided to use the isolate from hen 988 (Supplemental Fig. S10), subsequently called CVI-988 for further studies. This bird was part of a flock of chickens maintained under isolation at the CVI and free of ALV and MD. Although the origin of this flock is not mentioned in any of the publications or reports, this flock consisted of genetically highly MD susceptible line 7 chickens received from RPRL (Maas, personal communication, 2016). The positive results in laboratory challenge experiments prompted the team to take on much-larger-scale field trials. The first trials were initiated between January and June 1970 in the region Land van Weert, where previous flocks experienced 28%–32% MD losses. A total of 24 flocks comprising 160,000 1-day-old chicks were vaccinated resulting in less than 1% mortality (Boel, personal communication, 2016; 2,45,50) (Supplemental Fig. S11). In the meantime, Dr. Manfred Krasselt (Laboratory Dr. de Zeeuw, Gist-Brocades) conducted limited field trials in 1969–1970 comparing HPRS-16/Att with CVI-988 (Table 3) (2,47). Protection by CVI-988 was superior to that provided by HPRS-16/Att.

In view of the significant MD losses in the Netherlands, Rispens and Maas were pressured to start even-larger-scale experimental vaccinations. They were reluctant to do so because at that time insufficient field trials had been conducted for extensive statistical analysis and insufficient protection could lead to significant financial claims. After official support for extensive field trials was provided by the Dutch government (Maas, personal communication, 2013), 4,563,808 1-day-old chicks comprising 1070 parent and grandparent flocks were vaccinated between August 23, 1970 and April 20, 1971. Of the total number of flocks, 457 (1,977,074 chicks) provided sufficient data for statistical analysis. Only three of these flocks experienced economically significant MD losses (39,40).

At the time of these experiments I was being trained by Dr. Rispens in preparation for my work at the Instituto Nacional de Investigaciones Pecuarias of the Mexican Secretaria de Agricultura y Ganaderia in Mexico City, Mexico as part of the Dutch Technical Aid Program. I clearly remember the preparation of the vaccine with the use of 15-cm plastic Petri dishes and joining Rispens and Maas on at least one of the experimental vaccination days.

In addition to the vaccine production at the CVI, three Dutch vaccine companies received CVI-988 to help produce enough vaccine for the extensive field trials. Laboratory Dr. de Zeeuw (in 1988 acquired by Intervet, now MSD) and Philips-Duphar (now Zoetis) started commercial production of CVI-988 in 1972, and Intervet apparently did not produce CVI-988 until later. Interestingly, Rispens provided CVI-988 to these three vaccine companies without financial compensation to the CVI or the Dutch government. At that time the Dutch government had the official policy not to pursue patents for vaccine strains. This was not only the case for CVI-988, but also, for example, for the vaccine for foot and mouth disease developed by Dr. Frenkel of the CVI, which was provided to IFFA-Mérieux without financial compensation (Boel, personal communication, 2016). Times certainly have changed.

Based on the excellent protection, CVI-988 became the vaccine of choice for the Dutch poultry industry. Although IFFA-Mérieux received CVI-988 on October 26, 1973 (Gaudry, personal communication, 2016) and LAH some time in 1972 (Vielitz, personal communication, 2016), acceptance of CVI-988 by other countries did take considerable time for several reasons. The vaccine was not fully attenuated in the sense that it was still able to spread horizontally at p35, which was used as the vaccine passage (50). This raised concerns that it could cause problems in humans after accidental injection. In addition, von Bülow (56) reported that inoculation of genetically highly susceptible Rhode Island Red chickens with 10 times the vaccine dose caused gross neural lesions of the A- and B-type. In attempts to reduce the residual pathogenicity, de Boer *et al.* developed CVI-988 Clone C (28) and CVI-988 Clone C/R6 (29), but these two vaccines have not been widely used because the level of protection was not satisfactory.

When vvMDV strains appeared during the late 1970s, Vielitz noted that birds vaccinated with CVI-988 performed better in problem areas like Egypt than HVT-vaccinated birds (Vielitz, personal communication, 2016). When MD problems in HVT-vaccinated chickens occurred in Germany, CVI-988 was tested in commercial flocks under a provisional license, showing excellent results (55). LAH received the official license for the use of CVI-988 on November 5, 1985 (Vielitz, personal communication, 2016). Rhône-Mérieux received their license for France on June 28, 1989 (Gaudry, personal communication, 2016). Gaudry also played a key role in the importation of CVI-988 into the United States after

Rhône-Mérieux acquired Select. He convinced Drs. Dale King and Tom Mickle that this vaccine was needed in the United States (Gaudry, personal communication, 2016). After obtaining approval from APHIS, CVI-988 was provided to Witter, most likely by Mickle, on January 17, 1991. Shortly afterwards, Witter (60) and Witter *et al.* (70) showed that CVI-988 provide the best protection against challenge with vv+ strains of MDV. On June 29, 1994 Rhône-Mérieux (now Merial) received the license for the use of CVI-988 in the USA (Dr. Nikki Pritchard, personal communication, 2016). Twenty years after the introduction in the United States, CVI-988 remains the gold standard for licensed MD vaccines.

Unfortunately, Bart Rispens, born on May 10, 1929, passed away on November 11, 1973 and therefore never saw the worldwide application of his MD vaccine. In memory of his achievement, his collaborator and close friend, Henk Maas initiated the establishment of the Dr. Bart Rispens Award for the best publication in Avian Pathology over the 2-yr period preceding the Congress of the World Veterinary Poultry Association.

### SB-1, THE LUCKY STORY OF MY PH.D. DISSERTATION

As mentioned before, my introduction to MD research happened at the CVI in preparation for setting up a research team for MD at the Instituto Nacional de Investigaciones Pecuarias of the Mexican Secretaria de Agricultura y Ganaderia in Mexico City, Mexico. This project was funded by the Dutch Technical Aid Program, with technical advice provided by Rispens, who visited the Mexico project in the summer of 1971 after visiting RPRL in East Lansing. I was able to visit RPRL during the same time (Supplemental Fig. S12). I stayed with Bart Rispens and his wife, Will, in the house of Dr. Burmester, while the Burmesters were on vacation. It was in the Burmester residence that Bart and I worked hard on revising the two seminal papers on CVI-988 for Avian Diseases (49,50). In 1972, during my 4 yr in Mexico, I met Bruce Calnek at the AAAP meeting in New Orleans (1972) and again at the Western Poultry Disease Conference in Davis (CA) in 1974. The meeting in Davis ultimately led to the isolation of SB-1, because Calnek asked me if I was interested in becoming his Ph.D. student after his return from a sabbatical year at the HPRS in England. My contract with the Dutch government was ending July 1975 and the invitation to become a graduate student at Cornell University was very appealing. Located at the P.P. Levine Laboratory (Supplemental Fig. S13), I started my Ph.D. program at the end of August 1975 with a strong interest in studying how CVI-988 protected against challenge. However, in 1975 CVI-988 was maintained in the high-biosecurity facility of the CVI in Lelystad, where foot and mouth disease virus was used for research and vaccine production. As a consequence importation of CVI-988 had to go through the USDA high-biosecurity facility in Plum Island, NY for clearance, which was too expensive. This fact led me in a different direction.

Following the idea of Rispens that CVI-988-like strains could be present in nonvaccinated flocks free of any clinical disease, I searched for such a flock. Dr. Randall K. Cole (1912–2006), Professor Emeritus of the Department of Poultry Science, Cornell University mentioned that he had a flock of 28-wk-old S-strain chickens that were not vaccinated against MD and housed in building B of the poultry farm of the Department of Poultry Science (Supplemental Fig. S14). S-strain chickens with major histocompatibility complex B<sup>13</sup>B<sup>13</sup> were highly susceptible to MD (e.g., 53). Unfortunately this

chicken strain is no longer in existence. This particular flock was in full production and had no losses associated with MD. On October 14, 1975, Cole and I took blood samples from 15 hens for virus isolation in CKC. The initial passage showed some minor CPE changes in 3/15 samples, which upon passage showed numerous plaques. Positive cultures from bird C98 became the material for SB (S-strain chickens in building B). The second passage was inoculated into 1-day-old SPF S-strain chicks for the preparation of cell-free virus, which was used to inoculate CKC for biological cloning. The clone SB-1 was selected on August 3, 1976, and was subsequently propagated in CEF.

SB and SB-1 differed in several aspects from oncogenic strains such as the JM and GA isolates. The virus was growing much better in CEF than oncogenic strains such as JM and GA and CPE produced by SB and SB-1 in CKC were slightly different than CPE produced by the oncogenic viruses (51). As part of my Ph.D. thesis I wanted to address the following two key questions: 1) is SB-1 pathogenic and 2) can SB-1 induce protection against challenge? To address the first question SB-1 was injected into the amnio-allantoic cavity of 8-day-old embryos. This approach, previously used by Calnek *et al.* (17), increased the incidence of MD tumor lesions after inoculation with low-oncogenic strains such as CU-1 and CU-2. Inoculation of embryos with SB-1 never caused tumor lesions (19,51), but because degenerative lesions developed as a consequence of increased virus replication we used the term *nononcogenic* rather than nonpathogenic (51). This choice of nomenclature had important consequences when we filed for a patent (see below). The second question was addressed with challenge experiments showing that SB-1 did induce protection against challenge with oncogenic virus or with the JMV tumor transplant.

While we were working with SB and SB-1, von Bülow and Biggs (57,58) reported that the apathogenic HPRS-24 isolate was serologically different from oncogenic MDV and HVT. Their results led to the classification of three serotypes with pathogenic viruses grouped in serotype 1 and the apathogenic HPRS-24 and HVT placed in serogroups 2 and 3, respectively. We confirmed shortly afterwards that SB-1 and three other isolates (HN-1, GM-1, and JS-1) (22,74), previously classified as apathogenic, were also serotype 2 viruses (51).

During the early 1970s, Dr. Donald Zander, working at H&N Inc. (Redmond, WA), vaccinated chickens with freshly obtained blood from SPF chickens containing a mild MDV strain (HN-1). Blood was diluted 1:2 and inoculated subcutaneous within 24 hr of collection. Protection was rapidly induced by this approach. Interestingly, tissue-culture adaption of the virus resulted in attenuation of protection at p25, but the article does not provide details about the isolation and propagation of the virus (74). Partial vaccination of the flock resulted in adequate protection of all birds provided that management of the farm precluded early exposure (74). Rispens *et al.* (48,49) had a similar idea using vaccination with CVI-988 of 10% of a flock or adding 3-wk-old vaccinated shedder birds to 1-day-old chickens at a 1:10 ratio. However, the lack of control to early exposure with field strains of MDV made the success of this approach unreliable and it was never used extensively.

Cho and Kenzy (21) isolated a virus (HN) from broilers vaccinated with blood containing the HN-1 virus. These birds were transferred to isolation units immediately after vaccination. Blood samples for virus isolation were obtained at 4 wk of age and virus was isolated. P7 of HN was tested for pathogenicity by intra-abdominal inoculation of 3-day-old chicks with 16 and 166 plaque-forming units, respectively. At 5 wk postinfection 2/10 birds

developed limited gross visceral lesions. This finding became of crucial importance for the patent application of SB-1.

### THE PATENT STORY OF SB-1

When Calnek presented the information on the protection by SB-1 at the 49th Northeastern Conference on Avian Diseases at the University of Guelph (June 13–15, 1977), Dr. Dale King (Dr. Salsbury Laboratories, later Select and now Merial) suggested patenting the SB-1 strain. The idea was to use SB-1 in broiler breeders so that the offspring could be vaccinated with cell-free or cell-associated HVT without the presence of maternal antibodies to HVT. After some discussion between Bruce and me (I was somewhat influenced by Bart Rispens' idea about patents) we decided to file for a patent. A key factor in the ultimately successful application was the use of the term nononcogenic for SB-1. As mentioned before the nononcogenicity had been established by inoculation of 8-day-old embryos, which had not been done for other known serotype 2 strains such as HPRS-24 and HN. It was also fortuitous for us that HN caused tumors in a few birds (21). It has never been determined if these tumors were actually caused by HN or by a contamination with a serotype 1 MDV.

The patent attorney at Cornell University in 1978, Mr. Ralph Barnard, only worked under pressure, which was a concern for Calnek. The manuscript describing SB-1 had been submitted and accepted after some modifications. When the proofs were returned to the journal the patent had not yet been written. When Calnek called the journal he learned that we had 1 wk to write the patent before the publication would reach the Library of Congress. During that week, Barnard, Calnek and I, with the assistance of a law student, wrote the patent application. The document was sent to Washington, DC with the last commercial flight from Ithaca to Washington on the evening before the paper would be published. An attorney working for Cornell University received the document at the airport in Washington and hand delivered the document to the patent office just in time before the journal with the article (51) was deposited in the Library of Congress. The patent was granted on July 3, 1979 (Table 1) (Supplemental Fig. S15).

### THE USE OF BIVALENT VACCINES

Toward the end of the 1970s, reports from poultry producers in the United States suggested that HVT-vaccinated breeder and layer flocks experienced increased losses to MD. At the same time there was also an increase in condemnations of broilers because of skin leukosis, which is actually MD. Several research groups isolated MD strains with increased virulence such as RB-1B (52), Md5 and Md11, dubbed vvMDV, by Witter *et al.* (72). Neither HVT nor SB-1 were able to provide sufficient protection against the vvMDV strains. Independently of each other, Schat *et al.* (52) (Fig. 3) and Witter *et al.* (59) used the bivalent combination of HVT and SB-1 and found increased protection against challenge with vvMDV in laboratory experiments. Field trials in layers (20) and broilers (73) confirmed the efficacy of the bivalent vaccine. However, licensing for commercial use of SB-1 in the bivalent vaccine hit some roadblocks at the USDA. Dr. Bernard LaSalle, at the time Staff Veterinarian of the Veterinary Biologics Licensing and Standards, Animal and Plant Health Inspection Service of the USDA, did not want to allow any new MDV-associated vaccine without trials in monkeys to prove the absence of oncogenicity for mammalian species, a concern similar to that raised earlier by others for



Fig. 3. From left to right: Karel A. (Ton) Schat, W. (Bill) R. Shek, Bruce W. Calnek, and Julius Fabricant, circa 1981. Drs. Calnek, Fabricant, and Schat were involved in the field trials with the bivalent HVT + SB-1 vaccine at that time. The results were published in Reference 20. Shek was a graduate student at the time. Photo courtesy of Dr. Bruce Calnek.

CVI-988. However, under pressure from the poultry industry and Lasher, LaSalle was overruled and SB-1 was approved for use. Because SB-1 has slightly different characteristics than HVT, the vaccine industry initially had some problems with the propagation of the virus and representatives of the different vaccine manufactures came to the P.P. Levine Laboratory at Cornell University to learn how to propagate SB-1. At that time I could only accommodate 16 roller bottles, but that was sufficient to teach the proper technique for the propagation of SB-1. Select (now Merial) received the license for the use of SB-1 on May 17, 1985 (Pritchard, personal communication, 2016). I expect that other vaccine companies received the licenses around the same time.

The concept of bivalent vaccines was actually first used by Zander and Raymond (75) in an interesting way. As mentioned before, Zander's group originally used blood of SPF chickens infected with HN-1 to vaccinate all or part of the H&N flocks. When FC126 became more economical to use, the company switched to the use of FC126. In addition, 1%–5% of birds in each flock were also inoculated with HN-1 containing blood serving as seeder birds for the dissemination of HN-1 to the rest of the flock. This practice was discontinued at the end of 1976, but reinstated when vvMDV strains started to cause MD problems.

### THE FUTURE OF MD VACCINATION

When looking back at the early history of MDV vaccines, it is amazing to see what has been achieved, often with approaches that are now considered primitive. The evolution of MDV to increased pathogenicity in vaccinated birds raises the question of what will happen if the virus evolves to an even more virulent pathotype than the vv+ strains. When new vaccines are needed it seems unlikely that new strains will be isolated offering an improved protection over CVI-988. Witter and Kreager (69) actually raised the question of whether strains can be developed or isolated that exceed the efficacy of CVI-988. Perhaps molecular techniques may offer pathways to improved vaccines by deletions or alterations of specific genes, or perhaps by insertion of certain genes, but thus far this type of approach has not yielded commercial products.



Supplementary data associated with this article can be found at <http://dx.doi.org/10.1637/AVIANDISEASESJOURNAL-11429-050216-HistR.s1>

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