EXPERT REVIEWS

Vero cell platform in vaccine production: moving towards cell culture-based viral vaccines

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P Noel Barrett†, Wolfgang Mundt, Otfried Kistner and M Keith Howard

†Author for correspondence
Baxter BioScience, Biomedical Research Centre, Uferstraße 15, A-2304 Orth/Donau, Austria
Tel.: +43 120 100 4316
Fax: +43 120 100 4000
noel_barrett@baxter.com

The development of cell culture systems for virus propagation has led to major advances in virus vaccine development. Primary and diploid cell culture systems are now being replaced by the use of continuous cell lines (CCLs). These substrates are gaining increasing acceptance from regulatory authorities as improved screening technologies remove fears regarding their potential oncogenic properties. The Vero cell line is the most widely accepted CCL by regulatory authorities and has been used for over 30 years for the production of polio and rabies virus vaccines. The recent licensure of a Vero cell-derived live virus vaccine (ACAM2000, smallpox vaccine) has coincided with an explosion in the development of a range of new viral vaccines, ranging from live-attenuated pediatric vaccines against rotavirus infections to inactivated whole-virus vaccines against H5N1 pandemic influenza. These developments have illustrated the value of this cell culture platform in the rapid development of vaccines against a range of virus diseases.

Keywords: continuous cell line • emerging virus disease • influenza • rotavirus • smallpox • Vero • viral vaccine

One of the most important developments in the history of virus vaccine development was the demonstration of virus propagation in stationary cell culture. The demonstration by Enders, Weller and Robbins in 1949 that poliovirus could be grown in cell culture [1] was one of the key scientific discoveries that led to the development of poliovirus vaccines. In addition, the report that human viruses could be grown *in vitro* in a relatively safe and easy manner in monolayer cell cultures led to significant advances in virus vaccine development over the next 60 years. Prior to this development, the few available viral vaccines were produced in animal systems, such as calf skin for smallpox, rabbit spinal cord for rabies and mouse brain for Japanese encephalitis, or in embryonated eggs in the case of influenza and yellow fever viruses [2].

This work of Enders and colleagues was the precursor to major developments that allowed the development and manufacture of safe and effective vaccines against a number of devastating viral diseases. The first success was the growth of Lansing type II poliovirus in human cell monolayer culture [1]. These discoveries permitted Salk to grow large quantities of poliovirus in primary monkey testicular and kidney cells for the development of the inactivated poliovirus vaccine (IPV) [3]. Licensure of IPV was the first result of the cell culture revolution that permitted the development of many other viral vaccines. This first generation of cell culture vaccines utilized primary cell culture or cultures that had been subjected to a very limited number of subcultures owing to the very short lifespan of such cultures. The main sources of cells for primary culture were primates, chicken or duck embryos, hamsters and rabbits. All of these early primary cell culture systems suffered from the disadvantage of inconsistent starting material and concerns about contamination with a number of potential adventitious agents. In the early days of IPV and oral polio vaccine manufacture, these vaccines were contaminated with simian virus (SV)40, derived from primary Rhesus monkey kidney cells used in the manufacture [3]. It should be emphasized that all epidemiological studies carried out with the recipients of these vaccines demonstrated no negative safety impact of vaccination [4].

The next major development in cell culture for vaccine production was the use of human diploid cell lines for virus propagation to avoid the difficulties associated with the use of primary tissue culture. Diploid cell lines are defined as having a finite *in vitro* lifespan in which the chromosomes are paired (euploid) and are structurally identical to those of the species from which they

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were derived. In the early 1960s, scientists at the Wistar Institute selected the human diploid cell line WI-38 for the development of a cell culture-derived rabies vaccine [5,6]. This and other diploid cell lines, such as MRC-5, have been used for the manufacture of a number of vaccines, such as hepatitis A, IPV and rubella. These cell lines offer a number of advantages in that cell banks can be established, which can be screened for the absence of adventitious agents. However they suffer from the disadvantage that they can only be used for a restricted number of passages, as serial propagation results in senescence. In addition, they are not easy to use in large-scale production such as bioreactor technology using the microcarrier method. In general, they also need a more demanding growth medium and are difficult to propagate under serum-free conditions. The essential argument in favor of the use of diploid cell lines for the manufacture of human vaccines was the fact that they undergo senescence and are nontumorigenic.

By contrast, continuous cell lines (CCLs) for many years were not considered to be suitable substrates for the production of human medicinal products [7]. CCLs have the potential for an infinite lifespan, even though they may have been derived from a normal cell population and, as such, have tumorigenic potential. However, as new techniques in immunology, virology and molecular biology were developed, it became possible to examine viral vaccine cell substrates for characteristics of potential oncogenic properties in a more critical manner. Based on data from a number of studies, in 1986, a WHO Study Group considered a number of issues associated with the acceptability of new cell substrates for the production of biologicals [8]. They concluded that, in general, CCLs were acceptable for this purpose. However, it was noted that differences in the nature and characteristics of the products and manufacturing processes must be taken into account when making a decision on the use of a particular CCL in the manufacture of a given product. WHO requirements for CCLs used for biologicals production were published in 1987 [8]. In addition, the WHO Study Group recommended the establishment of well-characterized cell lines that would be of value to national control authorities and manufacturers of biologicals. In following up this recommendation, the WHO developed a master cell bank for Vero cells, a continuous cell line established from African green monkeys. This cell line was established in 1962 by Yasumura and Kawikata at the Chiba University (Japan) from the kidneys of a normal adult African green monkey [9]. The cell line was transferred to the US NIH at passage 93 and submitted to the ATCC by the NIH at passage level 113. It was then provided to the Institut Merieux at passage level 124 to generate the WHO Vero cell bank [10]. The reason for selecting this cell line was that it offered the immediate prospect of being utilized for a number of different vaccines being produced in other systems. A number of studies suggested that cells below particular passage numbers were not tumorigenic [10–14]. Tumorgenicity was defined as the inability to form progressively growing tumors in nude mice or immunosuppressed neonatal rats under conditions where HeLa cells generate invasive tumors. Collaborative studies in ten laboratories with respect to sterility, adventitious agents, tumorigenicity, presence of reverse transcriptase and identity showed that the WHO Vero cell bank met the WHO requirements for CCLs used for biologicals production [8]. In addition to updated WHO regulations [15], European [16], US [17] and International Conference on Harmonization (ICH) [18] regulations have now been published as a guidance for the screening of potential risks associated with the production of biologicals in CCLs, particularly contamination with adventitious agents, cellular DNA and growth-promoting proteins. Different laboratories have been using Vero cells with different lineages, which have been propagated under very different conditions. As such, it is essential to carry out a full characterization of the cell banks utilized for vaccine production, despite the established pedigree of Vero cells as a substrate for human vaccine production. Particular scrutiny has been attached to the safety issues associated with residual DNA and Vero cell protein. Although these are now considered to be impurities rather than tumorgenic agents, it is required that the purification process be validated to demonstrate the removal of cellular DNA to a level equivalent to not more than 10 ng per single human dose. This is the general requirement for injectable vaccines, unless otherwise prescribed. However, the fragment size of the residual DNA must also be characterized to ensure adequate degradation during the purification process steps. In addition, there is less concern about the DNA content in oral vaccines such as oral polio vaccine (OPV) and the rotavirus vaccines compared with vaccines administered by injection. Table 1 lists the results of a screening program for a Vero master cell bank recently carried out for the purpose of licensing a Vero cell-derived influenza vaccine, and which conforms with these various regulatory requirements.

Although a number of other CCLs, such as Madin-Darby canine kidney (MDCK) cells [19] and PER-C6 [20], are being considered or, in the case of MDCK, are currently being used in the development and manufacture of human vaccines, Vero cells are the most widely accepted CCLs by regulatory authorities for the manufacture of viral vaccines. Vero cells were first used for human vaccines with the production of IPV by Montagnon and colleagues at the Institute Merieux (Lyon, France) in the early 1980s [21], and this was followed by its use as an inactivated rabies vaccine. Vero cells have also been used for many years for the production of oral, that is, live poliovirus vaccine [22]. As such, there is over 25 years experience with Vero-derived human vaccines, with hundreds of millions of vaccine doses being distributed worldwide [23]. This experience has provided substantial evidence supporting the safety of this cell substrate, and has provided encouragement to further explore the use of this cell line for a range of different viral vaccines, which will be described later in more detail. These developments have been encouraged by the broad sensitivity of Vero cells to many viruses [24], which may be the result of the inherent defect with respect to interferon production by these cells. It was reported that Vero cells failed to produce interferon when infected with Newcastle disease, Sendai, Sindbis and rubella viruses, although the cells were sensitive to interferon. Under the same conditions, BSC-1 cells and other cells of primate origin produced interferon, indicating that this was a specific characteristic of Vero cells [25]. Another major advantage of the Vero cell line for vaccine production is that it can be grown and infected on microcarrier beads,

Table 1. Characterization of the Vero master cell bank.

Study/test	Test system	Result
Sterility	As defined by European Pharmacopoeia	Sterile
Mycobacterium tuberculosis	As defined by European Pharmacopoeia	Negative for the presence of *M. tuberculosis*
Mycoplasma	As defined by European Pharmacopoeia	Free from Mycoplasmas
Adventitious virus testing	*In vitro* assay in MRC-5, Vero and Chicken embryo cells	Negative, no adventitious viruses detected
	In vitro assay for detection of nonmurine Viral contaminants using MRC-5, Vero and MDCK cells	Negative, no adventitious viruses detected
	In vivo assay in suckling mice, adult mice, guinea pigs and eggs	Negative, no adventitious viruses detected
	Detection of CMV by cocultivation	CMV not detected
	HPV Panel	Negative for HPV types 6, 11, 16, 18, 31, 33 and 35 by RNA–DNA hybridization assay
	EBV detection by molecular hybridization	Negative for EBV DNA using southern blots and specific EBV probes
	EBNA test	Negative for EBNA
Bovine and porcine virus testing	Protocol for bovine virus detection according to 9CFR	Negative, no viruses detected
	In vitro assay for detection of porcine viral contaminants using PPK indicator cells	Negative, no viruses detected
Retrovirus testing	Reverse transciptase assay	Negative for both MN^{2+}- and Mg^{2+}-dependent reverse transcriptase assay
	Transmission electron microscopy for detection of virus, fungi, bacteria and mycoplasmas	No identifiable virus-like particles, mycoplasmas, fungi, yeast or bacteria detected
Identity	Isoenzymes analyses	Identity confirmed
	Nucleic acid fingerprinting	Identity confirmed
Cytogenic analyses	Chromosome frequency distribution	Heteroploid cells, chromosome frequency distribution corresponds to karyology of the repository reference seed stock (ATCC)
Tumorigenicity	*In vivo* tumorigenicity: tumor formation in nude (nu/nu) mice	No evidence for the presence of tumor formation
PCR tests for extraneous viruses	PCR tests for bovine viral diarrhea virus, porcine parvovirus, minute virus of mice and bovine polyomavirus	No virus sequences detected
PCR test for human and simian viruses	Detection of HIV-1/2, human T-cell lymphotrophic virus-1/2, hepatitis B virus, hepatitis C virus, human herpesvirus-6/7/8, simian foamy virus, simian immunodeficiency virus, simian virus-40, sugarcane mosaic virus, sapovirus, SAIDS retrovirus-1/2/3, simian T-cell leukemia virus and squirrel monkey retrovirus	No virus sequence detected

9CFR: Code of Federal regulations, Title 9 – Animals and Animal Products; ATCC: American-type culture collection; CMV: Cytomelagovirus; EBNA: Epstein-Barr nuclear antigen; EBV: Epstein–Barr virus; HPV: Human Papilloma virus; MDCK: Madin-Darby canine kidney; PPK: Primary pig kidney; SAIDS: Simian-acquired immunodeficiency syndrome.

and cultivated in fermenters to allow the large-scale production of vaccines. These developments were pioneered by Anton Van Wezel, who first demonstrated the high-density cell growth on microbeads for the production of polio and rabies virus vaccines [26]. This microcarrier technology has been further developed to allow the large-scale production of a number of vaccines using a

serum-free medium [27–29]. Such processes have been developed to allow amplification of a single 1-ml ampoule of cells to achieve a fully confluent microcarrier culture at a 6000-l scale within 8 weeks. This upscaling can be carried out without loss of cell productivity or viability with extremely consistent results. Table 2 demonstrates that cell density was not reduced during a typical scale-up process going from the 15- to the 6000-l scale. The maximum passage number that is achieved at the 6000-l scale with this process is 142. This amplification must be carried out with a cell bank, which will allow amplification to the 6000-l scale without exceeding a passage number, which is considered to have tumorgenic potential. Tumogenicity testing is also carried out at ten passage levels beyond this maximum-utilized passage number to ensure a large safety margin with respect to any theoretical safety concerns. Figure 1 also demonstrates the excellent consistency of these processes, with an almost identical cell metabolism being illustrated for 11 consecutive runs at the 6000-l scale, as measured by residual medium glucose concentration in a perfusion culture. These facilities can also be designed to allow the manufacture of a vaccine from wild-type highly pathogenic viruses, such as H5N1, by incorporating the appropriate biosafety level containment measures in the facility [30]. Following the development of polio and rabies virus vaccines 30 years ago, there was little further activity in Vero cell-derived vaccine development. However, in the last 5 years, there has been an explosion of activity, with a number of new vaccines having recently been licensed or are in late-stage development.

Recent developments in Vero cell-derived vaccines

Rotavirus

Two Vero cell-derived rotavirus vaccines have recently been licensed, and represent a dramatic improvement in the prospects of protecting infants against serious rotavirus infection after the withdrawal of an earlier vaccine, RotaShield® (Wyeth), owing to safety concerns, specifically an increased risk of intussusception [31].

Rotavirus is the leading cause of severe diarrheal disease in infants and young children, with 600,000 deaths occurring worldwide each year. The outer layer of the virus is composed of two proteins, VP4 and VP7, which define the different virus serotypes and represent the targets for neutralizing antibodies. The use of Vero cell cultures has been successfully applied in two different approaches to the development of a rotavirus vaccine.

Table 2. Consistency of Vero cell growth at different fermenter scales.

Days in culture	Cell density (x10^6/ml)/scale			
	15 l	*100 l*	*1000 l*	*6000 l*
1	ND	0.24	0.21	0.26
2	0.43	0.40	0.40	0.40
3	ND	0.68	0.79	0.77
4	1.27	1.16	1.30	1.24
5	ND	1.67	1.72	1.79
6	1.82	2.07	2.05	2.15
7	2.08	2.42	ND	2.73

ND: Not done.

A pentavalent live human–bovine reassortant virus, RotaTeq® (Merck), contains four reassortant rotaviruses expressing human VP7 protein (serotypes G1, G2, G3 and G4) and the VP4 protein (P7[5]) of the bovine virus, and one reassortant expressing the bovine virus VP7 protein (G6) and the human VP4 protein (P1A[8]) [32]. This vaccine was tested in a blinded placebo-controlled Phase III trial involving over 64,000 healthy, 6–12-week-old infants. Each subject received three oral doses at 4–10-week intervals [33]. Owing to adverse events associated with an earlier, tetravalent live vaccine based on human–rhesus rotavirus reassortants, the primary aim of the trial was to assess the safety of the vaccine with respect to intussusception, with the immunogenicity and efficacy of the vaccine also being evaluated. RotaTeq was shown to be safe and immunogenic, inducing group-specific serum IgA antibodies. During the trial, efficacy against severe G1–G4 gastroenteritis was 98%. The vaccine was licensed in the USA in 2006 and, to date, applications for licensure have been filed in over 100 countries [31].

A second Vero cell-derived rotavirus vaccine, Rotarix® (GlaxoSmithKline), was licensed in 2007. The vaccine is a monovalent, attenuated human rotavirus containing the most common of the human rotavirus VP4 and VP7 antigens, P1A[8] and G1 [34]. Attenuation was achieved by 26 passages in primary African green monkey kidney cells, followed by a further seven passages in serially passaged African green monkey kidney cells [35]. A randomized, double-blind, placebo-controlled Phase III clinical trial was carried out in over 63,000 healthy infants [36]. The infants received two oral doses at 2 and 4 months of age. The vaccine was shown to be safe, with no increase in intussusception, and provided 85% protection against severe rotaviral gastroenteritis. Although the vaccine is a monovalent vaccine based on a G1 strain, protection was demonstrated against the G1, G4 and G9 serotypes.

Flavivirus vaccines

Japanese encephalitis

Japanese encephalitis (JE) is a mosquito-borne, epidemic inflammatory disease of the CNS found across wide areas of South-East Asia. The maximum annual incidence was 50,000 cases, with a fatality rate of approximately 30% [37]. A formalin-inactivated vaccine produced from virus grown in mouse brains was developed in the 1930s, and the introduction of JE vaccine immunization programs after 1965 has gradually reduced the incidence of the disease in Japan, Korea and Taiwan. However, a high infection rate in pigs, the reservoir host, necessitates the continued use of vaccination [38]. Moreover, the area endemic for JE is expanding in Asia and, despite the efficient control by vaccination in Japan, China and Korea, the incidence of JE is increasing in Bangladesh, Burma, India, Nepal, Thailand, Vietnam and Indonesia [39,40].

However, despite demonstrating a clear efficacy, there are a number of disadvantages associated with the use of the mouse brain-derived vaccines. Owing to the high rate of systemic adverse

events associated with mouse brain impurities in the vaccine, travelers had been advised only to consider JE vaccination if the risk of infection was particularly high. To meet the needs of travellers, a new inactivated Vero cell culture-derived JE vaccine, Ixiaro® (Intercell), has been developed. The vaccine is based on an attenuated strain of JEV, SA14-14-2, which has been adapted to grow on Vero cells [41]. The purified finished product is adjuvanted with 0.1% aluminium hydroxide and contains neither the thimerosal preservative nor gelatin used in the mouse brain-derived vaccine (JE-VAX; Biken). The vaccine was tested for noninferiority in a double-blinded Phase III trial involving 867 subjects, and was shown to be both well-tolerated and immunogenic [41]. Two intramuscular injections of Ixiaro, 28 days apart, gave a seroconversion rate of 98%, compared with 95% given by three subcutaneous injections of JE-VAX [41]. A subsequent larger randomized double-blind placebo-controlled Phase III trial in 2675 subjects clearly demonstrated that the safety profile of Ixiaro was identical to that of the placebo control [42]. The vaccine has recently received EU licensure.

Parallel development of an inactivated JEV vaccine, called JE-PIV and based on the attenuated SA14-14-2 strain, is being undertaken jointly by the Walter Reed Army Institute of Research and the Cheil Jedang Corporation of South Korea [43]. A Phase II open-label unblinded study of JE-PIV, in which the vaccine was given as two or three intradermal injections, demonstrated that it was well-tolerated and could achieve 100% seroconversion [44]. In addition, a formalin-inactivated JE vaccine, based on the Beijing-1 strain of JEV and grown in serum-free vero cell microcarrier culture, is also in clinical development at Kaketsuken [45,46]. In a Phase I clinical trial, 60 young adult male subjects were given three immunizations with either a Vero cell-derived or mouse brain-derived JE vaccine. The Vero cell-derived vaccine was shown to induce adverse reactions in fewer subjects than the mouse brain-derived vaccine, and was statistically more immunogenic after three immunizations [46].

An alternative approach involving a Vero cell-derived, live attenuated JE vaccine has also completed Phase II clinical studies [40]. ChimeriVax™-JE (Acambis) is a chimeric yellow fever virus vaccine strain, 17D, which expresses the premembrane and envelope genes of the attenuated SA14-14-2 JEV strain [47]. Phase II studies showed that the chimeric vaccine was well-tolerated, and immunization resulted in a 94% seroconversion rate in the 87 subjects receiving a single dose of ChimeriVax-JE. Interestingly, a booster immunization on day 30 gave no increase in the immune response to JEV [48].

Dengue fever

Dengue fever is a mosquito-borne viral disease with clinical manifestations varying from a mild influenza-like infection to infrequent cases of hemorrhagic fever and lethal shock. Annually, approximately 100 million new infections occur in subtropical and tropical regions worldwide [49]. In spite of multiple attempts since the 1930s to develop Dengue vaccines, no licensed vaccine is available to date. One reason for this situation is the existence of four antigenically related serotypes that necessitate the use of a tetravalent vaccine capable of inducing a balanced immunity between serotypes, since multiple serotypes can cocirculate, and the circulating serotypes can vary from one season to the next [50]. Another concern is the risk that antibody-dependent immune enhancement of infection by one serotype could be caused by antibodies to another serotype.

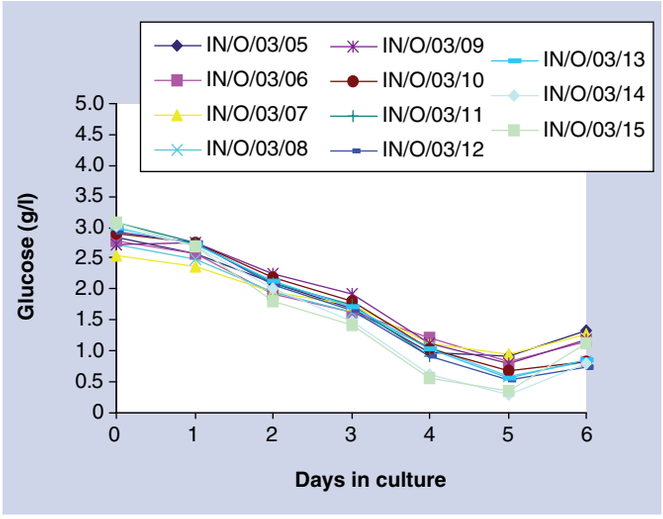

Figure 1. Residual glucose concentration in Vero cell perfusion culture of 11 consecutive runs at the 6000-l scale.

Two Vero cell-based live-attenuated vaccines currently under development are thought to represent the best approach for safe and effective vaccination against dengue virus infection [50]. Both vaccines are based on chimeric flaviviruses. Advantages of live-attenuated dengue virus vaccines over the equivalent killed vaccines include the low cost, the possibility of rapid immunization during epidemics and induction of long-lasting immunity similar to that found after natural infection, which should reduce the risk of inducing lethal hemorrhagic fever/shock syndrome.

One candidate vaccine, developed by the Bloomberg School of Public Health and the NIH, is based on chimeras using a mutated Dengue serotype (DEN)-4 background [51]. The RNA genome of the original wild-type DEN-4 virus was transferred into a bacterial plasmid and engineered to introduce an attenuating 30-nucleotide deletion in the 3′-untranslated region. The mutated positive-sense RNA genome was transcribed *in vitro* and transfected directly into Vero (WHO) cells in serum-free medium to recover infectious virus. Chimeric viruses were constructed by the replacement of the attenuated DEN-4 virus E protein with that of DEN-1, DEN-2 or DEN-3 [52,53]. Testing of the monovalent DEN-1 vaccine in a Phase I human trial demonstrated that the vaccine was well-tolerated and immunogenic after a single subcutaneous vaccination. A seroconversion rate against DEN-1 of 95% was achieved [54]. A Phase I dose-finding study with the DEN-2 chimera demonstrated that this vaccine was also well-tolerated and achieved 100% seroconversion in the vaccinated group [55]. In a placebo-controlled Phase II trial of the nonchimeric attenuated DEN-4 vaccine, the vaccine was considered well-tolerated and immunogenic, with 95–100% of

vaccinated subjects seroconverting [56]. However, elevated liver enzyme levels were noted in the serum of subjects receiving the highest vaccine dose. Consequently, DEN-4 was further attenuated by the introduction of a mutation that restricted the growth of the candidate vaccine virus in human hepatocarcinoma cells. A placebo-controlled Phase I trial demonstrated that the modified vaccine was well-tolerated without inducing either a detectable viremia or an elevation of liver enzymes [57]. Immunization with the highly attenuated vaccine did, however, result in 100% seroconversion and this virus is now considered to be the lead candidate for inclusion in the final tetravalent vaccine.

The development of a chimeric dengue vaccine based on the ChimeriVax platform (Acambis), as described previously for JE, is also being pursued [58]. A Vero cell-based tetravalent vaccine consisting of four chimeric strains with E-proteins representing each of the four serotypes carried on the 17D attenuated yellow fever virus backbone is also in clinical development [59]. In a Phase II clinical trial in 66 volunteers, two vaccinations of the tetravalent chimeric vaccine, administered 6 months apart, were well-tolerated and immunogenic. A seroconversion rate of 67% was achieved against at least three serotypes after only one vaccination [50]. Phase III trials are now planned with this tetravalent chimeric vaccine.

West Nile encephalitis

The ChimeriVax platform is also being exploited for the development of a vaccine against West Nile virus (WNV) infection. Although first isolated in Uganda in 1937, it was not until the 1990s that the virus has emerged as a serious threat to humans, horses and domestic bird flocks [60]. The first WNV outbreak in the USA was recorded in New York state in 1999 and the infection spread rapidly, both across North America and into the Caribbean and South America [61–63].

ChimeriVax-WN02 is the most advanced WNV vaccine in development and, as with the other ChimeriVax vaccines, is cultivated in Vero cells. The vaccine is a chimera expressing the protective West Nile E-protein on an attenuated 17D yellow fever virus vaccine backbone. In addition to the use of the attenuated yellow fever backbone, three additional attenuating mutations were introduced into the WNV envelope protein sequence, further increasing the safety of the vaccine [64]. A randomized, double-blind, placebo-controlled study in 80 healthy 18–40-year-old adults has been performed [64,65]. The ChimeriVax-WN02 vaccine was well-tolerated and resulted in a 97% seroconversion rate after a single vaccination. IFN-γ-secreting T cells were also detected in up to 87% of the trial subjects receiving the ChimeriVax-WN02 vaccine. Phase II trials in humans have been initiated with a single-dose immunization regimen [101].

In addition, a nonadjuvanted inactivated wild-type WNV vaccine, WN-Vax, produced in Vero cell cultures, is in development at the National Institute of Infectious Diseases (Tokyo, Japan) [66]. In preclinical testing, the inactivated vaccine resulted in 100% seroconversion in all immunized animals. Importantly, no interference was seen in animals with pre-existing immunity to Japanese encephalitis virus, a serologically related flavivirus.

Vaccines for emerging virus diseases & potential bioterrorism agents

Traditionally, the development of new vaccines against exotic viral infections has been in the realm of the military, with the aim of protecting servicemen posted in tropical regions of the world [67]. However, the rapid increase in airline traffic and the continued encroachment of human activities on environments harboring novel zoonotic viruses has greatly increased the risk of epidemics in human populations. In addition, more recently, the possibility that such agents could be used as the basis of a weapon has also been considered [68]. A dramatic example of how rapidly and devastatingly a novel disease agent can spread, even by person-to-person contact, was provided by the severe acute respiratory syndrome (SARS) outbreak in late 2002 caused by a previously unknown coronavirus [69]. Similarly, a rapid expansion of areas endemic for a virus, such as occurred with WNV, can be facilitated by insect transmission and by the infection of highly mobile reservoir species, such as birds. In the face of such threats, the well-established use of Vero cell culture for the manufacture of a range of viral vaccines (Table 3) provides a strong platform on which new vaccines can be developed.

Ross River fever & Chikungunya fever

Ross River virus (RRV) is an *Alphavirus* causing an infectious disease characterized by arthritis, often accompanied by a fever and rash. It is endemic in many parts of Australia, with approximately 5000 cases being recorded annually [70]. However, in 1979–1980, an explosive epidemic of Ross River fever occurred in several Pacific nations [71]. An inactivated candidate vaccine (Baxter) against RRV produced in animal protein-free Vero cell microcarrier fermenter cultures was demonstrated to be immunogenic and capable of inducing a protective immune response in a mouse challenge model [72]. This vaccine is currently in a dose-finding Phase I/II clinical trial in 400 healthy adults.

Recently, a closely related *Alphavirus* of the Semliki forest complex, chikungunya virus (CHIKV), has caused a large epidemic of fever and incapacitating polyalthralgia with severe muscle pain across islands of the Indian Ocean and the Indian subcontinent [73]. Owing to the popularity of the region with tourists, CHIKV was imported into many Western countries, particularly France and the USA [74]. Moreover, in Italy, a local transmission cycle was briefly established following the introduction and subsequent dispersal of a vector capable of transmitting CHIKV – that is, the Asian tiger mosquito (*Aedes albopictus*) – in the 1990s [75] . Many of these recent chikungunya epidemics were associated with mutations in the virus genome that enhanced transmission in the *A. albopictus* mosquito, endemic to the region. This recent epidemic was of particular note and concern because it was demonstrated that, unlike previous epidemics, the outbreak of chikungunya fever in 2005–2006 was associated with fatalities at a rate of one per 1000 infections [76].

In response to the recent epidemic and the continued threat of expansion of the disease into new areas, two Vero culture-based vaccines are currently in preclinical development. An inactivated

Table 3. Vero cell-produced vaccines against viral diseases.

Study (year)	Disease	Vaccine type	Genus	Ref.
Wang *et al.* (2008)	Chikungunya fever	Live attenuated	*Alphavirus*	[77]
Howard *et al.* (2008)	Chikungunya fever	Inactivated	*Alphavirus*	(Howard *et al.*, Unpublished Data)
Guirakhoo *et al.* (2004) Blaney *et al.* (2007) Blaney *et al.* (2008)	Dengue fever	Live attenuated or live chimeric	*Flavivirus*	[59] [52] [53]
Tauber *et al.* (2007) Tauber *et al.* (2008) Srivastava *et al.* (2001)	Japanese encephalitis	Inactivated	*Flavivirus*	[41] [42] [43]
Kuzuhara *et al.* (2003) Guirakhoo *et al.* (1999) Monath *et al.* (2003)	Japanese encephalitis	Live attenuated (or chimeric)	*Flavivirus*	[46] [47] [48]
Vesikari *et al.* (2006) Ruis-Palacios *et al.* (2006)	Viral gastroenteritis	Live attenuated	*Rotavirus*	[33] [36]
Montagnon (1989)	Polio	Live attenuated	*Picornavirus*	[92]
Montagnon (1989)	Polio	Inactivated	*Picornavirus*	[92]
Montagnon (1989)	Rabies	Inactivated	*Lyssavirus*	[92]
Kistner *et al.* (2007)	Ross River fever	Inactivated	*Alphavirus*	[72]
Spruth *et al.* (2006) Qu *et al.* (2005) Qin *et al.* (2006)	Severe acute respiratory syndrome	Inactivated	*Coronavirus*	[28] [78] [79]
Monath *et al.* (2004)	Smallpox	Live attenuated	*Orthopoxvirus*	[80]
Lim *et al.* (2008)	West Nile encephalitis	Inactivated	*Flavivirus*	[66]
Monath *et al.* (2006)	West Nile encephalitis	Live attenuated	*Flavivirus*	[64]
Kistner *et al.* (1998) Ehrlich *et al.* (2008)	Influenza	Inactivated	*Orthomyxovirus*	[27] [30]

vaccine against CHIKV (Baxter) is being developed based on the Vero cell culture process developed previously for influenza vaccines [27].

An alternative, live-attenuated chimeric vaccine is in preclinical testing at the University of Texas (TX, USA) with the aim of developing an effective vaccine that can be manufactured cheaply in endemic countries [77]. Three candidate chimeric vaccines were developed by incorporating the structural proteins of chikungunya into three different *Alphavirus* vectors (based on strains of Sindbis virus, Venezuelan equine encephalitis virus or Eastern equine encephalitis virus [EEEV]). The rationale for this approach is that the attenuated phenotype of such chimeras might be more stable than the attenuation achieved by the traditional method of multiple passages in tissue culture. The chimeric viruses were found to be capable of growth to high titers in Vero cell culture, and were shown to be attenuated in animal models of chikungunya infection. In addition, the chimeric viruses were shown to be both immunogenic and protective in a mouse challenge model. Of the three chimeric viruses tested, the vaccine based on the EEEV vector backbone (BeAr436087 strain) is considered to have the most suitable safety profile for use in humans since, in addition to the attenuation imposed by the chimeric nature of the recombinant virus genome, South American strains of EEEV such as BeAr436087 are not known to cause disease in humans [77].

Severe acute respiratory syndrome

Severe acute respiratory syndrome emerged in China in late 2002 and spread rapidly around the world [69]. The epidemic resulted in over 8000 infections and almost 800 deaths. Although there have been no reports of community-acquired human SARS infections since January 2004, a re-emergence of the disease and the concomitant threat on global health is still possible, as the virus is still circulating in populations of reservoir animals. SARS therefore provides a strong incentive for the rapid development of a candidate vaccine. A candidate vaccine has been produced on Vero cells using the animal protein-free Vero cell microcarrier culture system (Baxter). The double-inactivated (formalin plus ultraviolet irradiation), whole-virus vaccine was found to be highly immunogenic and efficacious in preclinical models, inducing neutralizing antibodies and SARS CoV-specific IFN-γ- and IL-4-secreting T cells. The vaccine also conferred protection against high-titer live virus challenge in a mouse model [28]. A Phase I clinical trial in healthy adults is planned for 2009. Similar formalin-inactivated and β-propriolactone-inactivated whole-virus vaccines are in preclinical development [78,79] and, in addition, Sinovac has completed Phase I clinical trials with an inactivated SARS vaccine. The placebo-controlled clinical trial tested two doses of vaccine in groups of 12 adults aged between 21 and 40 years of age. Two immunizations resulted in 91.6 and 100% seroconversion in the high- and low-dose groups, respectively [102].

Smallpox

The WHO declared in 1980 that smallpox had been eradicated. However, there is a growing concern that the variola virus could be used as a biological weapon, since immunity to the virus will have decreased with the cessation of vaccination. In response, the US government has been replacing the original DryVax® smallpox vaccine stockpile that was originally established as a

measure against an unexpected re-emergence of smallpox. A Vero cell culture-grown smallpox vaccine, ACAM2000 (Acambis), was chosen as the basis of the new stockpile because the original method used to produce the DryVax material (i.e., lymph collected from the skin of live calves or sheep) is now considered incompatible with good manufacturing practice [80]. This Vero cell-derived vaccine was developed from a single biological clone derived from the DryVax vaccine. It was first isolated and passaged on MRC-5 cells before passaging on Vero. In a series of clinical trials through to Phase III involving a total of 3851 subjects, the Vero cell-derived vaccine was shown to be highly immunogenic, inducing seroconversion in up to 96% of subjects previously naive to vaccinia. The reactogenicity profile of the new vaccine was found to be identical to that of the original DryVax vaccine.

Influenza

Licensed inactivated vaccines for influenza have been available since the 1940s. The current vaccines are trivalent and contain an H1N1 and an H3N2 subtype of the influenza A strains and an influenza B strain. However, to date, all such vaccines are produced in embryonated chickens' eggs. This is a cumbersome process that requires the coordinated supply of hundreds of thousands of eggs during a manufacturing run. The lack of reliable supplies of high-quality eggs results in limitations in the amount of vaccine that may be produced, and creates a concern that there is a real risk of major shortfalls in vaccine supply in the event of a pandemic, when the demand for vaccine would be much higher. There is also evidence that the selection of human influenza viruses for high-yield growth in eggs is also associated with the selection of antigen variants that may be suboptimal for inducing protective antibodies to wild-type virus circulating in humans [81–83]. In contrast to influenza viruses grown in eggs, virus propagated exclusively in mammalian-derived tissue culture has been reported to be representative of the natural virus [84]. Studies in ferrets have also demonstrated that an inactivated influenza vaccine grown in MDCK cells induced higher mean serum hemagglutination inhibition (HI) and neutralizing antibody titers than egg-grown vaccine, and induced superior protection against subsequent challenge with infectious virus grown in either cell type [85].

The use of Vero cell fermenter culture technology, which involves a well-characterized and controlled, scaleable, closed manufacturing process, avoids the disadvantages described for the manufacturing process based on eggs. Although initial studies found Vero cells to be a poor substrate for the growth of influenza viruses [86,87], work with a bank of serum protein-free medium-adapted Vero cells demonstrated that high yields of all influenza A and B strains tested could be achieved with the addition of exogenous trypsin to activate the hemagglutinin protein on the surface of the virus [27]. The Vero cell manufacturing process has been scaled up to 6000 l and Phase III clinical trials of the trivalent split-seasonal influenza vaccine are underway (Baxter).

In addition to seasonal epidemics, influenza pandemics occur at unpredictable intervals. It is widely believed that the continued spread of highly pathogenic strains of H5N1 influenza in wild and domestic bird populations, plus the often fatal transmission from infected birds to man, makes this virus the most likely source of the next influenza pandemic. Most pandemic influenza vaccines produced to date are based on attenuated reassortant viruses. The reassortants are generated using the hemagglutinin and neuraminidase genes of the circulating wild-type virus, and the other six genes of the human influenza H1N1 strain A/Puerto Rico/8/34, a strain normally providing high virus yields in embryonated chicken eggs. The H5N1 virus is attenuated by the deletion of the polybasic cleavage site, which confers virulence by greatly expanding the range of host cells for the virus [88,89]. Such reassortants are then subjected to extensive safety testing by the WHO before being distributed to the vaccine manufacturers. This procedure is essential to allow the production of the virus under biosafety level 2-enhanced containment, which is the highest biosafety level available to large-scale egg-based manufacturing facilities. However, the generation and safety testing of new reassortants takes several weeks, and would result in a delay in the delivery of a pandemic influenza vaccine. Moreover, the vaccine provides an optimal antigenic fit with the circulating wild-type virus only with respect to the hemagglutinin and neuraminidase genes, and not with the remaining six genes that are derived from the H1N1 A/Puerto Rico/8/34 strain.

Vero cell technology provides particular advantages for the rapid production of a vaccine against pandemic influenza. Unlike embryonated chickens' eggs, Vero cells allow the high-titer growth of wild-type H5N1 influenza strains under biosafety level 3-enhanced conditions, the level required by the WHO for wild-type H5N1 [90]. Consequently, wild-type virus seed stock can be obtained and immediately expanded in preparation for the manufacture of vaccine. In addition, Vero cell cultures make the production of the pandemic influenza vaccine independent of the supply of embryonated chicken eggs. This is particularly important if there were to be a major shortfall of eggs in the event of a pandemic, particularly in a situation where chicken flocks have been depleted by infection with highly pathogenic influenza virus.

The use of Vero cell culture technology for the production of vaccines against pandemic influenza therefore avoids the delay and potential antigenic mismatch associated with vaccine production using egg-adapted, reverse genetics-derived attenuated virus. The process is amenable to the production of consistent inactivated influenza virus vaccines at a 6000-l manufacturing scale [91]. For vaccine production, the virus harvest is inactivated using a highly stringent procedure involving two separate steps: formalin and ultraviolet (UV) treatment. Formalin alone is sufficient to achieve total inactivation with a large safety margin, as confirmed by stringent safety assays of the bulk vaccine in two highly susceptible cell systems, that is, Vero and chicken embryo cells. The double-inactivated virus is then purified by continuous sucrose gradient centrifugation followed by ultra-/dialfiltration steps prior to formulation. To date, eight strains of H5N1 influenza virus, including clade 0, 1, 2.1 and clade 2.2 isolates, have been grown to high titer in Vero cell culture (Kistner O *et al.* Unpublished Data). This underlines the reliability of the Vero cell culture system for the production of vaccines to H5N1 influenza, and provides confidence that it will be possible to produce such vaccines to new H5N1 variants as they arise.

Candidate vaccines have been developed using both the clade 1 strain A/Vietnam/1203/2004 and the clade 2.1 strain A/Indonesia/05/2005 [103] based on a nonadjuvanted, whole-virus formulation. The candidate vaccine based on the A/Vietnam/1203/2004 clade 1 strain has been tested in Phase I/II [30] and Phase III studies, and has been shown to be well-tolerated and highly immunogenic at low doses (7.5 µg hemagglutinin). The vaccine induces high-titer neutralizing antibodies not only against the clade 1 vaccine strain, but also against a range of heterologous clade 0, 2.1, 2.2 and 2.3 strains. Licensure was recently obtained in Europe for this first cell culture-derived H5N1 vaccine (Baxter).

Expert commentary & five-year view

Following the licensure of a Vero cell-derived live smallpox and two Rotavirus vaccines in the USA in 2006 and 2007, it is anticipated that a range of new inactivated and live virus Vero cell vaccines will be licensed in the next few years. Substantial advances have been made in the most technologically challenging area, that is, influenza vaccines. Phase III studies are currently underway for Vero cell-derived seasonal influenza vaccines in the USA, and a Vero cell-derived H5N1 vaccine has recently been licensed in Europe. These are especially significant developments for Vero cell vaccines, as influenza viruses are known to grow poorly in cell culture without the addition of specific activating enzymes required to cleave the hemagglutinin.

Owing to the previous low yields obtained in cell culture, influenza vaccines have continued to be produced by growth in embryonated eggs, a procedure that has remained almost unchanged for the past 50 years. However, this is a cumbersome procedure, which is particularly susceptible to microbial contamination, and the supply of vaccine in the past has been endangered owing to large-scale failures in manufacturing. In addition, this production technology is completely dependent on the supply of chicken eggs, which could be endangered in the event of an influenza pandemic caused by an avian virus that is highly pathogenic for chickens. The development of cell culture technologies such as Vero will have a significant impact in securing influenza vaccine supply, particularly in the event of an influenza pandemic.

The technology platform associated with these developments is now being utilized for a range of inactivated whole-virus vaccines and it is anticipated that vaccines against emerging viral diseases such as SARS, Ross River, West Nile and Chikungunya will be licensed or enter into late-stage development over the next 5 years. The Vero platform should prove particularly valuable in rapidly responding to such emerging threats with new vaccines. The susceptibility of the cell line to a wide range of viruses increases the probability that high virus yields can be obtained with newly emerging viruses. This was best illustrated by the unexpected finding that the SARS coronavirus grows to high titers in Vero, allowing preclinical development of a candidate vaccine within an 18-month timeframe [28]. The ability to use wild-type virus for product development is also a major advantage associated with this platform. The Vero microcarrier technology can be readily established in secure facilities, which can be operated under enhanced biosafety level 3, the containment level that is required for working with a number of highly pathogenic viruses. As such, wild-type virus can be used for vaccine development and manufacturing without the requirement of developing attenuated strains, which would be needed for manufacturing platforms, such as embryonated eggs, where this enhanced biosafety level cannot be achieved on a large scale. This ability to work with wild-type virus further reduces the development time for vaccines against emerging viral diseases.

In addition to novel vaccine development, it is possible that existing vaccines, which are currently manufactured using primary cell culture, could be converted to a Vero-derived platform. For example, a number of manufacturers still produce polio vaccine using primary cells derived from primates. Vaccines such as tick-borne encephalitis and yellow fever, which are produced in primary chick cell culture and embryonated eggs, respectively, could be produced in high yields in Vero. However, the high costs of building new facilities and conducting extensive clinical bridging studies may inhibit such developments for existing licensed vaccines. On the other hand, it is being recognized that regulatory opinion has changed totally in the last 30 years with respect to the risks and benefits of primary culture compared with CCLs. The theoretical risks of tumorigenicity associated with cell lines such as Vero have been abolished by the development of new testing technologies, whereas the introduction of screening technologies such as PCR have demonstrated the theoretical risks of contamination of primary cell lines with a range of adventitious viruses. Such regulatory considerations, together with the multitude of advantages associated with the Vero platform, will continue to drive vaccine development and production using this cell line.

Financial & competing interests disclosure

All authors are employees of Baxter Healthcare, a vaccine manufacturer. They are all holders of company stock and stock options. P Noel Barrett, Wolfgang Mundt and Otfried Kistner are holders of patents concerning vaccine manufacture in Vero cells. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Key issues

- Continuous cell lines are gaining acceptance from licensing authorities.
- The Vero cell line is the most widely accepted continuous cell line.
- Vero cells are highly susceptible to infection with a multitude of different viruses.
- Novel Vero cell-derived vaccines against major pathogens such as rotaviruses have been licensed.
- Vaccines against a range of emerging viral diseases such as West Nile, sever acute respiratory syndrome and Chikungunya are in development using the Vero platform.
- Development of Vero cell-derived influenza vaccines is a major advance in pandemic preparedness.

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Affiliations

• P Noel Barrett
Baxter BioScience, Biomedical Research Centre, Uferstraße 15, A-2304 Orth/Donau, Austria
Tel.: +43 120 100 4316
Fax: +43 120 100 4000
noel_barrett@baxter.com

• Wolfgang Mundt
Baxter BioScience, IZD-Tower, Wagramerstraße 17–19, A-1220 Vienna, Austria

• Otfried Kistner
Baxter BioScience, Biomedical Research Centre, Uferstraße 15, A-2304 Orth/Donau, Austria

• M Keith Howard
Baxter BioScience, Biomedical Research Centre, Uferstraße 15, A-2304 Orth/Donau, Austria