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MINIREVIEW

Multivalent and Multipathogen Viral Vector Vaccines

Katharina B. Lauer,[a,d] Ray Borrow,[a,b] Thomas J. Blanchard[a,c]

University of Manchester, Institute of Inflammation and Repair, Manchester, United Kingdom[a]; Vaccine Evaluation Unit, Public Health England, Manchester Royal Infirmary, Manchester, United Kingdom[b]; Consultant in Infectious Diseases and Tropical Medicine, Royal Liverpool Hospital, Liverpool, United Kingdom[c]; University of Cambridge, Department of Pathology, Cambridge, United Kingdom[d]

ABSTRACT The presentation and delivery of antigens are crucial for inducing immunity and, desirably, lifelong protection. Recombinant viral vectors—proven safe and successful in veterinary vaccine applications—are ideal shuttles to deliver foreign proteins to induce an immune response with protective antibody levels by mimicking natural infection. Some examples of viral vectors are adenoviruses, measles virus, or poxviruses. The required attributes to qualify as a vaccine vector are as follows: stable insertion of coding sequences into the genome, induction of a protective immune response, a proven safety record, and the potential for large-scale production. The need to develop new vaccines for infectious diseases, increase vaccine accessibility, reduce health costs, and simplify overloaded immunization schedules has driven the idea to combine antigens from the same or various pathogens. To protect effectively, some vaccines require multiple antigens of one pathogen or different pathogen serotypes/serogroups in combination (multivalent or polyvalent vaccines). Future multivalent vaccine candidates are likely to be required for complex diseases like malaria and HIV. Other novel strategies propose an antigen combination of different pathogens to protect against several diseases at once (multidisease or multipathogen vaccines).

KEYWORDS multidisease vaccine, multipathogen vaccine, multivalent vaccine, polyvalent vaccine, viral vector vaccine

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Address correspondence to Katharina B. Lauer, katharina.lauer@postgrad.manchester.ac.uk.

In the last 20 years, many new human diseases have emerged, and worryingly, diseases previously presumed to be under control, such as diphtheria, plague, and polio, have resurged (1, 2). Vaccines are recognized to be one of the most cost-effective interventions for the prevention of infectious diseases. Effective and safe vaccines capable of undergoing mass production provide the prospect of eradication of certain diseases. In most cases, a large proportion of vaccination scheme costs arises from maintaining cold chains, storage, and transport as well as the salaries of medical and paramedical staff rather than the costs of the vaccines themselves. Despite this, some recently introduced advanced vaccines are markedly more costly. The expenditure on vaccination programs can be minimized by the well-established practice of combining individual vaccines (e.g., diphtheria/tetanus/pertussis or mumps/measles/rubella), but each component has to be manufactured separately and the method of combination can be complex. With the advent of genetically engineered viral vaccines, it has become feasible to combine multiple protective antigens into a single viral vector, e.g., a complex filovirus-vesicular stomatitis virus (VSV) recombinant (3). This is especially true of larger viral vectors (e.g., herpesviruses, poxviruses, and adenoviruses [AdVs]), where there are few restrictions imposed by gene packaging limits. It is increasingly recognized that viral vectors may be deployed to protect against a broad range of infectious diseases, for example, protozoal (e.g., malaria [4]) and mycobacterial (e.g., tuberculosis [5, 6]) infections. This allows the prospect of multipathogen vaccines, in

which a single vaccine agent can be envisaged to simultaneously protect against several common global pathogens, employing well-established and safe vectors in a cost-effective and unified program. Further, the underlying principles of genetic engineering and vaccine design may be applied to the prevention of infections, where the problem of protection of the host from multiple serotypes or genotypes has to be addressed. It is also important to recognize that eliciting protective cytotoxic T lymphocyte responses to epitopes within conserved viral proteins is another means to the same end (7).

Viral vector vaccines have been applied extensively in veterinary medicine (although these will not be discussed in detail in this minireview). An outstanding example of this is Raboral V-RG (Merial), the first oral live vaccinia virus (VacV) vector vaccine expressing the glycoprotein (GP) of Evelyn-Rokitnicki-Abelseth rabies virus. Before its introduction, rabies control in wildlife relied mostly on depopulation and the vaccination of individual animals. Raboral V-RG allowed oral vaccination on a large scale using vaccine-containing baits. Several countries have used Raboral V-RG safely without any adverse effects and have achieved complete rabies control (8, 9). The hurdles that have to be taken for introducing viral vector vaccine candidates into humans are appreciably higher than those for animals. Thus, the development of human viral vaccines and their licensing remain behind those achieved with animal vaccines. The pressure to find vaccines for diseases causing widespread epidemics, such as the recent Ebola outbreak, has accelerated efforts to fast track viral vaccine candidates in humans. Promising examples like the Ebola vaccine based on a recombinant adenovirus and modified vaccinia Ankara (MVA) may mark the advent of the first of a new generation of viral vaccines used in humans (10–13).

The development of new combined vaccines requires reflection on the terminology that is currently used to describe this new class of chimeric vaccines. A review of relevant literature reveals a problematic ambiguity in definition. The term "multivalent/polyvalent vaccine" is ambiguously used to describe either a vaccine candidate with the ability to protect against several diseases or a vaccine candidate that can protect against several strains of a single pathogen. In general parlance, "multivalent/polyvalent" refers to an agent that is effective against different types of the same organism. In accordance with this terminology, an infection consisting of multiple pathogens is generally described as a multipathogen disease or simply multidisease. To avoid confusion, the following nomenclature is proposed to distinguish the different types of combined vaccines.

DEFINITIONS

Multivalent/polyvalent vector vaccine. Combined antigens from different strains (serotypes/serogroups) of one pathogen in a single vector to immunize against one disease.

Multidisease/multipathogen vector vaccine. Key protective antigens from two or more pathogens in a single vector to immunize against several diseases.

Figure 1 shows a schematic overview of a multivalent/polyvalent and a multipathogen/multidisease viral vector.

In this minireview, we will detail existing multipathogen and multivalent vaccines derived from viral vectors. We will draw a distinction between simple multipathogen vaccines where the vector itself forms a part of the protective agent (for example, recombinant vaccinia viruses with the theoretical but currently irrelevant potential to induce immunity to smallpox) and those where the vector backbone is a vehicle to deliver protection to two or more additional pathogens.

Another possibility to generate multipathogen or multivalent vaccines is based on vectors where protective antigens have been replaced by antigens derived from heterologous viruses. While these vaccines are likely to yield protection to the heterologous virus, the protection yielded against the vector agent is likely to be impaired. These vaccine candidates will not be discussed further in this minireview. We will also compare and contrast the larger viral vectors that are likely to serve as backbones for

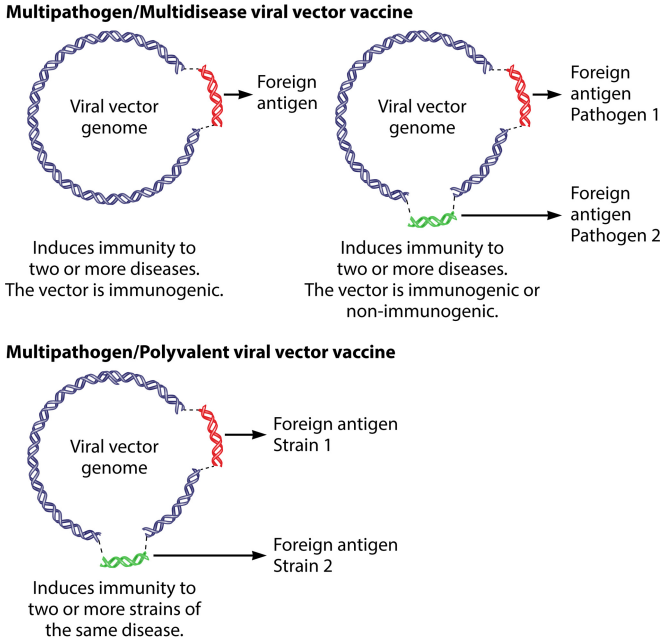

FIG 1 Schematic overview of multipathogen/multidisease and multivalent/polyvalent viral vector vaccines.

future broad-spectrum multipathogen and multivalent vaccines and address the technological hurdles remaining before such human vaccines become widely distributed. Table 1 provides an overview of the current multivalent and multipathogen viral vector vaccines that are under investigation.

MULTIVALENT AND MULTIPATHOGEN VACCINES FOR HUMAN APPLICATIONS

A wide variety of viruses have been investigated as single-pathogen vector vaccines; remarkably fewer have been used for multivalent and multipathogen applications. Viruses have to meet several requirements to be considered suitable multipathogen or multivalent vectors. The viral vector has to be capable of taking up large fragments of immunogenic genes, together with regulatory elements (e.g., promoter, polymerase, terminator, etc.), or immunomodulators like cytokines, to enhance humoral and cellular immune responses (14–16). These need to be expressed efficiently and stably, sometimes from different loci within the genome and preferably without the persistence of the recombinant virus in the host or its integration into the host genome. Other factors that have to be considered when choosing a vector platform are the lack of toxicity in the host, affordable large-scale production, or issues with preexisting vector immunity that may lead to a reduced immune response to the vector (15, 16). Table 2 compares the viral vectors discussed in this article with respect to their individual vector characteristics and suitability as multivalent/multipathogen viral vector candidates.

Measles virus. Among viral vector platforms, measles virus (MV) is a promising candidate. Measles virus, an exclusively human pathogen, is an enveloped virus in the family of *Paramyxoviridae* with a single-stranded, negative-sense RNA genome. Several MV strains (e.g., the Moraten, Schwarz, or Edmonston measles virus vaccine strain) have been safely used as vaccines for many years; they exhibit strong immunogenic properties leading to lifelong protection. MV replication occurs strictly in the cytoplasm of infected cells, which contributes to a consistent safety profile because no viral DNA is integrated into the host's genome (17). The ability to achieve the stable insertion of more than 5,000 nucleotides into the MV genome (unlike other RNA viruses), together with the efficient expression of transgenes and low production costs, makes MV a

TABLE 1 Overview of multivalent/polyvalent and multipathogen/multidisease viral vector vaccine candidates

Vector	Disease(s)[a]	Immunogenic insert(s)	Insertion site	Animal model	Administration[g]	Cellular immune response	Humoral immune response	Challenge(s)[g]	Protective immunity[a]	Reference(s)
Adenovirus	Marburg virus, Ebola virus	NP ZEBOV, GP EBOV, GP SEBOV, GP Ci67, GP Ravn, GP Musoke, NP Musoke	Between the ITR[f]	Cynomolgus macaques	i.m., 4×10^{10} total PFU (day 0, day 63)		✓	1st challenge (wk 0): s.c. MARV Musoke or i.m. ZEBOV (1×10^3 PFU); 2nd challenge (wk 10): i.m. SEBOV or s.c. MARV Ci67 (1×10^3 PFU)	100% of animals protected	36
	Ebola virus*	GP SEBOV, GP ZEBOV	Between the ITR	Mice (C57BL/6; BALB/c)	i.p. or s.c., 10^8 PFU (wk 0, wk 16, wk 24)	✓	✓	i.p., 1×10^3 PFU mouse-adapted ZEBOV-derived Ebola virus	100% of animals protected	32
	Influenza*	HA of A/Indonesia/05/05 and A/Vietnam/1203/04	Into the E1 region	Mice (BALB/c)	i.m., 1×10^8 PFU	✓	✓	i.n., 100 MID_{50} of influenza virus Egypt/08, TK/VA, G1/99, pH1N1, or X-31	Provided the same HA subtype was included in vaccine, the challenge virus was neutralized	82
		HA of A/Netherlands/219/03 and A/Chicken/Hong Kong/G9/97	Into the E1 region	Mice (BALB/c)	i.m., 1×10^8 PFU	✓	✓	i.n., 100 MID_{50} of influenza virus Egypt/08, TK/VA, G1/99, pH1N1, or X-31	Provided the same HA subtype was included in vaccine, the challenge virus was neutralized	
	Marburg virus*	GP fusion protein of MARV Ci67, Ravn, and Musoke strains	Between the ITR	CD-1 mice	i.p., 1×10^8 PFU (wk 0, wk 8)		✓			83
				Guinea pigs	s.c.; low dose: 5×10^6 PFU; medium dose: 5×10^7 PFU; high dose: 5×10^8 PFU (wk 0, wk 4)		✓	s.c. with MARV Musoke, Ravn, or Ci67 (2×10^3 LD_{50})	Low dose group: 83.3%; medium/high dose group: 100% of animals protected	
Coxsackievirus group B	Myocarditis	Hexon protein L1 loop of AdV2	Between capsid protein P-1D and protease P-2A	Mice (BALB/c)	i.p., 5×10^5 $TCID_{50}$ (a: day 0; b: day 0, day 14; c: day 0, day 14, day 28)		✓			55
Human parainfluenza virus type 3	Measles, respiratory tract infection	HA of MV Edmonston	Between N/P, P/M, or HN/L genes	Golden Syrian hamster	i.n., 1×10^6 PFU		✓	Wild-type PIV3 (1×10^6 PFU)	100- to 1,000-fold reduction of PIV3 virus titers	49
Measles virus	Measles, hepatitis B	HBsAg and/or HBcAg (ayw type)	Between M/P genes (HBsAg), H/L genes (HBcAg)	Mice ($IFNAR^-$, CD46)	MV-HBsAg i.n. or i.p., 5×10^5 PFU		✓			84
	Measles, HPV-associated disease	L1 protein	Between P/M genes	Mice ($IFNAR^-$, CD46)	i.p., 1×10^5 PFU (day 0, day 28)		✓			20
	Measles, HIV	Env gene gp140/gp160 (HIV-1 isolate, 89.6), V3 loop deletion	Between P/M or H/L	Mice ($IFNAR^-$, CD46)	i.p., 15×10^6 $TCID_{50}$	✓	✓			85
	Measles, HIV	F4 antigen (= p24 [BH10], RT [HXB2], Nef [Bru-Lai], and p17 [BH10]) of HIV-1 subtype B	Between P/M genes	Mice ($IFNAR^-$, CD46)	i.p., 1×10^4 or 1×10^7 $TCID_{50}$	✓	✓			86
				Cynomolgus monkeys	i.m., 1.6×10^4 $CCID_{50}$/ml (a: day 1; b: day 1, day 29, day 57	✓	✓			
	Measles, HIV	Gag-Pol fusion protein and gp140dCFI (e1), or Gag-Pol fusion protein and gp140dV1/2dCFI (e2) of HIV-1 clade B	G-P between H/L gene and e1/e2 between P/M	Mice ($IFNAR^-$, CD46)	i.p., 1×10^4 or 1×10^5 PFU (wk 0, wk 4)	✓	✓	i.n. pseudochallenge using 5×10^6 PFU recombinant vaccinia virus-HIV-gag	Immunized animals were less susceptible to weight loss	87

(Continued on following page)

TABLE 1 (Continued)

Vector	Disease(s)[a]	Immunogenic insert(s)	Insertion site	Animal model	Administration[g]	Cellular immune response	Humoral immune response	Challenge(s)[g]	Protective immunity[a]	Reference(s)
	Measles, HIV	F4 antigen (= p17, p24, RT and Nef) of HIV-1C		Cynomolgus macaques	i.m., 1×10^4 $TCID_{50}$ and 1×10^5 $TCID_{50}$ MV1-F4 (day 0, day 84)	✓	✓			88
	Measles, West Nile virus	WNV[c] E protein (IS-98-STI)	Between P/M genes	Mice (IFNAR$^-$, CD46)	1×10^4 or 1×10^6 $TCID_{50}$ (wk 0, wk 4)		✓	i.p., LD_{50} of WNV IS-98-ST1	100% of animals protected	89
				Mice (BALB/c)	2 μl pooled immune sera from IFNAR$^-$ CD46 mice		✓	i.p., $10 \times LD_{50}$ of WNV IS-98-ST1	100% of animals protected	
	Measles, SARS[b]	Spike protein (SARS-CoV[d], specimen no. 031589)		Mice (IFNAR$^-$, CD46)	i.p., 1×10^5 $TCID_{50}$ (day 0, day 28)		✓	i.n., 1×10^5 PFU SARS-CoV	100% of animals protected	90
	Measles, SARS	Nucleocapsid protein, spike glycoprotein (SARS-CoV Urbani)	Between P/M genes	Mice (IFNAR$^-$, CD46)	i.p., 1×10^4 PFU (wk 0, wk 4, or wk 8)		✓			91
	Measles, dengue fever	EDIII from E protein (aa[e] 295 to 394) and the ectodomain of the M protein (aa 1 to 40) from strain FGA/89 French Guiana for serotype DV1, Jamaica/N.1409 for DV2, DV3 H87, 63632/76 Burma for DV4	Between P/M genes	Mice (IFNAR$^-$, CD46)	i.p., 1×10^5 $TCID_{50}$ (wk 0, wk 4)		✓			92
	Measles, chikungunya fever	C, E3, E2, 6K, E1 (La Reunion strain 06-49)		Mice (IFNAR$^-$, CD46)	i.p., 1×10^3, 1×10^4, or 1×10^5 $TCID_{50}$ (wk 0, wk 4)		✓	i.p., 1×10^2 PFU CHIKV 06-49 (= 33 times LD_{50})	100% survival (immunized with 10^4 or 10^5 $TCID_{50}$); 83% survival (immunized with 10^3 $TCID_{50}$)**	26, 27
Modified vaccinia Ankara	Influenza H5N1 strains*	HA of A/Vietnam/1203/04, A/Indonesia/CDC669/06, and A/Anhui/01/05	Deletion site III	Mice (BALB/c)	i.m., 100 μl 8×10^7 $TCID_{50}$ (day 0, day 28)		✓	i.n., 10 MLD_{50} of A/Vietnam/1203/04 or A/chicken/Shanxi/2/06	100% of animals protected	61
Varicella-zoster virus	Mumps, chickenpox/shingles	HN and F protein (or F protein with serine 195 replaced with tyrosine)	Unique long domain	Guinea pigs	s.c., 2×10^6 to 5×10^6 MRC-5 cells infected with VZV vOka-HN-F (day 0, day 14, day 28, day 42)		✓			42

[a]An asterisk indicates a multivalent/polyvalent vaccine candidate. Two asterisks indicate a vaccine candidate that advanced to phase 1 clinical studies.
[b]SARS, severe acute respiratory syndrome.
[c]WNV, West Nile virus.
[d]SARS-CoV, severe acute respiratory syndrome coronavirus.
[e]aa, amino acid.
[f]ITR, inverted terminal repeat.
[g]i.m., intramuscularly; i.p., intraperitoneally; s.c., subcutaneously; i.n., intranasally; $CCID_{50}$, 50% cell culture infective dose; MID_{50}, 50% median infective dose.

TABLE 2 Comparison of the viral vectors discussed in this minireview based on specific vector characteristics

Vector	Virus group	No integration into the host genome	Replication in the cytoplasm	Insertion capacity[a]	Induction of cellular and humoral immunity	Preexisting vector immunity has proven problematic
Adenovirus	dsDNA	+	−	++	+	+
Coxsackievirus group B	ssRNA	+	+	+	+	+
Measles virus	ssRNA	+	+	++	+	−
Modified vaccinia Ankara	dsDNA	+	+	+++	+	−
Parainfluenza virus 3	ssRNA	+	+	++	+	−
Varicella-zoster virus	dsDNA	+	−	+++	+	−

[a]+++, up to 10 to 30 kb; ++, up to 4 to 8 kb.

valuable potential vaccine delivery system (18, 19). In many multipathogen vaccine candidates based on measles virus vectors, the virus itself is used as an immunogen.

One example is a MV vector (Moraten Berna measles vaccine strain sequence) human papillomavirus (HPV) vaccine candidate (rMVb2-HPV-L1), generated by Cantarella and colleagues (20), which proved to induce strong humoral immune responses against MV and HPV in transgenic interferon alpha receptor-deficient (IFNAR$^{-/-}$) CD46 mice. Reverse genetics technology enabled the rescue of MV (an RNA virus) from cloned plasmid DNA, containing MV antigenomes, in cell culture using the human helper cell line 293-3-46. The structural L1 protein sequence from HPV16, found to be immunogenic in previous studies, was inserted between the M and P sequence of the MV, forming virus-like particles after expression (20–22). The stability of the transgene expression, an important factor for a successful vaccine candidate, was tested over 10 passages in MRC-5 cells (human fetal lung fibroblasts) and showed no reduction. Furthermore, recombinant rMVb2-HPV-L1 did not exhibit a reduced growth kinetic compared to that of the "empty" MV. The immunogenic activity of rMVb2-HPV-L1 was evaluated in a murine immunization study with MV-susceptible mice (IFNAR$^{-/-}$ CD46; devoid of the interferon type I receptor plus expression of human CD46). Mice were injected intraperitoneally with rMVb2-HPV-L1 or the parental MV at day 0 and 4 weeks later with 10^5 PFU. All of the mice that were immunized with rMVb2-HPV-L1 mounted L1-specific humoral immune responses, which is comparable to the humoral immune response elicited in mice after three intramuscular injections with the standard HPV vaccine (Cervarix; GlaxoSmithKline Biologicals). Cervarix is a licensed virus-like particle vaccine against disease associated with HPV16 and HPV18, where virus-like particles are obtained with a baculovirus expression system (23). The serum of immunized mice was also examined for anti-measles virus antibodies. No difference in the immune response to MV was observed with the recombinant virus compared to that of the parental MV. As MV has the capacity for larger inserts (5 to 6 kb), this vector may be exploited for expressing immunogens of other HPV types (e.g., HPV18). Additionally, the authors suggest the insertion of the E6 or E7 proteins, which may allow the vaccine candidate to be used for immunotherapy (20).

In another approach by Brandler et al. (26), a recombinant measles vaccine expressing chikungunya virus-like particles was generated using a helper cell line rescue approach (24). Chikungunya virus (CHIKV) is an alphavirus with a positive RNA genome and is transmitted by mosquitoes especially in Southeast Asia, Africa, and the Indian subcontinent. In recent years, there has been a tendency for the virus to spread to more temperate regions. The measles virus Schwarz strain was used for the insertion (between the phosphoprotein and matrix gene of MV) of the C, E3, E2, 6K, and E1 structural protein sequences of CHIKV La Reunion strain 06-49, which accounts for most epidemics worldwide (25). Transgenic CD46-IFNAR mice, susceptible to measles infection, were injected intraperitoneally with two consecutive doses (ranging from 10^3 to 10^5 50% tissue culture infective dose [$TCID_{50}$]) 1 month apart. The control group received empty MV Schwarz strain. All mice vaccinated with MV-CHIKV showed the generation of specific antibodies for the MV vector and CHIKV as well as specific cellular immune responses (interferon gamma [IFN-γ] enzyme-linked immunosorbent spot [ELISPOT]

assay on splenocytes), which were boosted after the second immunization. Furthermore, a plaque reduction neutralization test showed that CHIKV-neutralizing antibodies were induced. A challenge study, 1 month after the last injection, with 100 PFU of CHIKV 06-49 (equal to 33 times the 50% lethal concentration [LD_{50}] by intraperitoneal injection) was performed. All of the mice that were immunized with 10^4 or 10^5 $TCID_{50}$ of MV-CHIKV were completely protected from CHIKV even when there was preexisting immunity to MV. Of the mice that were immunized with 10^3 $TCID_{50}$ of MV-CHIKV, 83% survived the lethal challenge, and all of the control mice injected with the MV Schwarz strain developed disease and died. In addition, antibodies elicited by the MV-CHIKV vaccine candidate showed neutralizing activity against other clinical isolates (La Reunion 2006, India 2011, Congo 2011, and Thailand 2009) in plaque reduction neutralization tests. Furthermore, passively transferred immune serum from MV-CHIKV-vaccinated mice protected five out of six mice against a lethal challenge with CHIKV (100 PFU of CHIKV 06-49) (26). The MV-CHIKV vaccine candidate was further evaluated in a randomized, double-blind, placebo-controlled phase 1, dose-escalating study including an active comparator (Priorix; GlaxoSmithKline Pharma GmbH, Vienna, Austria; live virus vaccine against measles, mumps, and rubella). The measles strain in the Priorix vaccine is homologous to the MV used to design the MV-CHIKV vaccine candidate. Healthy adults received a low dose (1.5×10^4 $TCID_{50}$ per 0.05 ml), medium dose (7.5×10^4 $TCID_{50}$ per 0.25 ml), or high dose (3.0×10^5 $TCID_{50}$ per 1 ml) of MV-CHIKV suspended in HEPES buffer with ammonium sulfate, Priorix, or placebo (sterile saline) on day 0 and 28 (the placebo was administered on day 90) or on day 0 and 90 (the placebo was administered on day 28). The geometric mean titers of neutralizing antibodies in the blood of participants were lower in the low dose and Priorix vaccine groups than in the medium and high dose groups. Nevertheless, all groups showed 100% seroconversion after booster immunization. Further, the impact of preexisting anti-measles immunity on the MV-CHIKV vaccine candidate was investigated and was found to have no impact on the performance of the vaccine candidate. The authors conclude that a phase 2 clinical trial is warranted to evaluate this promising vaccine candidate further (27).

Adenovirus. Adenoviruses (AdVs) have been widely studied as vectors for gene therapy and vaccines targeting various diseases, such as malaria or hepatitis C. Several characteristics make them attractive as vaccine vectors, including manufacturability and the ability to elicit broad immune responses. Adenoviruses are double-stranded DNA (dsDNA) viruses that replicate in the nuclei of vertebrates. They are easily manipulated into taking up foreign DNA (up to 8 kb) and can be cultivated in several cell types (dividing and nondividing cells as well as dendritic cells) to produce high virus titers and high levels of protein expression (19, 28). Expression levels can even be enhanced by using heterologous promoters. Another advantage of AdV is their ability to induce strong T-cell responses, including cytotoxic T cells. However, preexisting vector immunity—as is present in a large proportion of individuals—inhibits efficient expression of transgenes and inactivates the viral vector. Potential alternatives are nonhuman AdV vectors or engineered vectors. Adenoviral vectors are available as replication deficient and competent for mammalian cells (29–31).

The generation of multivalent Ebola virus (EBOV) vaccines employing an AdV platform was pioneered by Wang and colleagues (32). They created a bivalent complex adenovirus-based vaccine (cAdVax) vector, utilizing complex adenovirus technology and carrying the GP of Ebola virus Sudan (Boniface strain, SEBOV) and Zaire (Zaire-95 strain, ZEBOV). The cAdVax vectors are replication-defective adenovirus vector platforms with deleted E1, E3, and E4 genes, enabling the vector to accommodate large amounts of foreign DNA (33–35). BALB/c and C57BL/6 mice were immunized intraperitoneally with 1×10^8 PFU of the bivalent vaccine candidate at 0, 16, and 24 weeks. The $cAdVaxE_{S/Z}$ vaccine candidate was able to induce SEBOV and EBOV Ebola-specific antibodies to both strain responses as well as cell-mediated immune responses. To investigate if the $cAdVaxE_{S/Z}$ vaccine candidate protects mice from a lethal challenge

with a mouse-adapted Ebola virus strain (ZEBOV-derived), BALB/c and C57BL/6 mice were immunized by subcutaneous injection with 1×10^8 PFU of the bivalent vaccine candidate on day 0 and 35 followed by a challenge with 1,000 PFU of mouse-adapted Ebola virus (intraperitoneally). All vaccinated animals were 100% protected (32).

On the basis of this work, Swenson and colleagues developed a multistrain filovirus vaccine utilizing complex adenovirus technology and an adenovirus vector (cAdVax) (36). *Filoviridae*, primarily Ebola virus and Marburg virus, cause severe disease in humans and nonhuman primates. The high divergence between these species and the lack of cross-protection make vaccine development a difficult task (37). This pan-filovirus vaccine approach comprised antigens of Zaire Ebola virus (ZEBOV), Sudan Ebola virus (SEBOV), and the Ci67, Ravn, and Musoke strains of the Marburg virus (MARV). Four different cAdVax vectors were designed, the EBO2 vector containing two copies of the nucleoprotein (NP) of ZEBOV, the EBO7 vector expressing glycoproteins (GPs) of EBOV and SEBOV, the M8 vector expressing the Ci67 and Ravn GPs, and the M11 vector expressing the Musoke GP and NP genes. All four vectors were portioned equally (1×10^{10} PFU) into one combination vaccine, which was then injected intramuscularly into cynomolgus macaques on day 0 and with a booster vaccination after 63 days. The vaccinated animals were challenged with 1,000 times the lethal dose of MARV followed by EBOV or vice versa. All vaccinated primates were 100% protected against ZEBOV and SEBOV as well as the three Marburg virus species (Ci67, Ravn, and Musoke) (36).

Concordant with the above studies, Pratt and colleagues reported a multivalent Ebola virus vaccine candidate (EBO7) based on the cAdVax system, which expressed glycoproteins of SEBOV (Boniface strain) and ZEBOV (Kikwit strain) (38). Furthermore, the M8-recombinant-containing (36) Marburg virus Ci67 and Ravn GP, which was designed in the above study, was included. Immunization of cynomolgus macaques with an equal mixture of M8 and EBO7 (1×10^{10} PFU administered intramuscularly on day 0 and boosted on day 65 or 120) led to similar levels of antibodies against ZEBOV and SEBOV. The simultaneous administration of both vaccine candidates (M8 and EBO7) did not interfere with the levels of antibodies generated against ZEBOV or SEBOV. To test whether the bivalent vaccine candidate protects the vaccinated animals from Ebola virus disease, the macaques were challenged (intramuscularly) with 500 PFU of ZEBOV, 800 PFU of SEBOV, or 800 PFU SEBOV followed by 1,100 PFU ZEBOV. All vaccinated macaques survived the lethal challenge without developing signs of disease. The authors further investigated whether the EBO7 vaccine candidate confers protection against aerosol challenge with 900 to 1,000 PFU of aerosolized SEBOV and 100 to 500 PFU of aerosolized SEBOV after vaccination with 1×10^{10} PFU of EBO7 (day 0 and 71). The vaccine protected the vaccinated macaques after aerosol challenge with either virus in an otherwise lethal dose, even with preexisting vector immunity to the adenoviral vector (38). There is no data on the M8 recombinant, which was administered simultaneously with the EBO7. It would be interesting to investigate whether coadministration protects macaques from a challenge with the respective Marburg virus species. Based on the results reported by Swenson and colleagues (36), M8-vaccinated macaques survived a challenge with 1,000 times the lethal dose of MARV; it seems likely that the authors are suggesting that similar protection against MARV may be achieved, but this needs to be investigated in the future. The administration of multiple viral vectors simultaneously has to be evaluated carefully. Viral vectors harbor the potential risk of unintended recombination events (e.g., with other viral vector vaccines or naturally occurring viruses) in the host, which may lead to hybrid species with unknown characteristics. The use of replication-defective vectors reduces this risk but may not eliminate it completely.

Varicella-zoster virus. Varicella-zoster virus (VZV) is endemic worldwide, with infection rates as high as 90% before adolescence. The first vaccines against VZV were developed as early as 1984. Currently used vaccines (ATC codes J07BK01 and J07BK02; http://www.whocc.no/atc_ddd_index/?code = J07BK&showdescription=no) consisting

of live attenuated VZV have been monitored for years and have demonstrated a high safety profile with only minor side effects in healthy people (39, 40). Besides this, the host range of VZV is restricted to humans, obstructing uncontrolled environmental spread. The safety and the opportunity to insert and maintain large DNA inserts, combined with the ability to induce strong cellular and humoral immunity, sparked interest in using it as a recombinant vaccine candidate for generating multivalent/multipathogen vaccines (40, 41).

Matsuura and colleagues reported a successful application of VZV as a vaccine vector by introducing the two major surface proteins of the mumps virus (MuV) (hemagglutinin-neuraminidase [HN] and fusion protein) into the VZV vOka strain using a bacterial artificial chromosome (BAC) system (42). The integral membrane protein HN is responsible for receptor binding on host cells and MuV neuraminidase and hemagglutinin activity, whereas the F protein's main activity lies in viral penetration and hemolysis. The recombinant vOka-HN-F exhibited reduced growth kinetics with atypical cytopathogenic effects and syncytium formation leading to cell detachment in MRC-5 cells (human fetal lung fibroblast cells). These effects were overcome by introducing a S195Y mutation (serine-to-tyrosine substitution) to prevent membrane fusion (vOka-HN-F-S195Y). Guinea pigs were immunized four times (subcutaneously) with 2×10^6 to 5×10^6 MRC-5 cells infected with either vaccine candidate in 2-week intervals. Both vaccine candidates induced neutralizing antibodies against the VZV vector, and the animals immunized with VZV vOka-HN-F mounted slightly higher titers. Further, neutralizing antibodies against MuV were induced (higher in the group vaccinated with vOka-HN-F-S195Y). Only slight differences in the immune responses of both recombinant constructs were observed (42). These data suggest that VZV is a strong candidate for future multivalent or multipathogen vaccine development. It may be advised to use this viral vector cautiously, as it is replication competent in the human host.

Human parainfluenza virus 3. Human parainfluenza virus 3 (PIV3) is a member of a group of four parainfluenza viruses in the family *Paramyxoviridae*.

Parainfluenza viruses are nonsegmented negative-strand RNA viruses with a genome size of roughly 15,000 nucleotides, which can be easily manipulated or attenuated by reverse genetics (see also MV). The viral replication takes place in the cytoplasm of the host with no need for integration into the genome. Recombination events are rare in PIV, which contributes to the stability of inserted transgenes (43, 44).

As PIV3 is the second leading cause of hospitalization for viral respiratory tract disease, the development of a PIV3 vaccine is encouraged, particularly in combination with an already established vaccine to facilitate implementation into routine vaccine schedules.

The parenterally administered standard MV vaccine harbors the risk of being neutralized during the first months of life by serum antibodies passively transferred from mother to baby. A new MV vaccine candidate bypassing this would be a valuable asset in infant vaccination schedules. Therefore, a combined vaccination strategy, including PIV and MV, has been suggested. The viral backbone was generated from wild-type PIV3 through recovery from plasmid-borne cDNA using recombinant DNA technology (45). Further, attenuated versions of wild-type PIV3 have been developed (e.g., PIV3*cp*45) and have shown promising results as vaccine candidates (46–48). In this study, the hemagglutinin (HA) protein of the MV (Edmonston strain) was inserted into PIV3 or attenuated PIV3 between the N and P genes, P and M genes, or HN and L genes. These vaccine candidates induced antibodies against MV and PIV3 in golden Syrian hamsters, especially when inserted into the N-P or P-M junction. No significant difference in neutralizing antibodies was observed when using attenuated PIV3 compared to that when using wild-type virus. A challenge experiment with wild-type PIV3 that took place 28 days after intranasal immunization with 10^6 PFU of a vaccine candidate conferred significant protection to viral replication in the respiratory tract. Previous studies in monkeys suggest that PIV3 is able to replicate efficiently even in the presence of passively acquired PIV3 antibodies, leading to the conclusion that even with preex-

isting maternally MV antibodies the intranasally administered MV-PIV3-HA vaccine may be protective.

The authors report that their recombinant PIV3-MV vaccine candidate elicits, on average, five times more serum antibodies than the licensed live attenuated measles virus vaccine that is administered by intramuscular injection. It is also suggested to employ antigenic serotypes of PIV3 (e.g., PIV1 or PIV2) for prime boost vaccinations to reduce the risk of vector immunity and efficient transgene expression (49).

Coxsackievirus group B. Coxsackieviruses are positive-sense single-stranded RNA (ssRNA) viruses. Six serotypes of group B coxsackievirus (CVB1 to CVB6) have been described, of which CVB3 has been particularly identified as a potential vaccine vector. All CVBs incorporate four capsid proteins and seven nonstructural proteins and include two proteases. Although the small genome has a somewhat limited capacity to stably integrate foreign genetic material, CVBs are interesting viral vector candidates because strong immune responses (cellular and humoral) are generated following an infection (50). Coxsackieviruses are known to cause gastrointestinal distress, myocarditis, or dilated cardiomyopathy in humans. Together with CVB, only human adenovirus type 2 (AdV2) has been regularly linked to heart disease (51–54). To date, no vaccines against either virus are commercially available to protect from human heart disease. Hofling and colleagues (55) investigated a chimeric CVB3 vaccine candidate expressing the antigenic L1 loop of AdV2 hexon protein (the L1 loop of AdV2 has produced promising results in a rabbit model before [56]) from a locus between the capsid protein P-1D and the protease P-2A in the CVB3 genome. The inserted sequence was expressed over 10 passages in HeLa cells; however, a virus species corresponding to the parental strain was detected in passages 8 and 10, indicating that the recombinant virus is unstable. Reduced virus titers in comparison to the parental CVB3 strain were observed, suggesting that the insertion leads to an attenuation of the viral vector. To investigate the potential of the CVB3-Ad2L1 vaccine candidate to generate a humoral immune response, murine immunogenicity studies were performed. BALB/c mice were injected (intraperitoneally) with 5×10^5 $TCID_{50}$ of CVB3-Ad2L1 once, twice, or three times in 2-week intervals. The results showed that the CVB3-Ad2L1 vaccine candidate was able to induce anti-CVB3 and anti-Ad2 hexon L1 loop-neutralizing and -binding antibodies (titers increased with the number of booster injections). Interestingly, preexisting anti-CVB antibodies boosted the immune response further and led to even higher levels of anti-Ad2 antibodies in mice after receiving three injections of the multipathogen vaccine candidate. An evaluation of the CVB-Ad2L1 immune response over time to determine the duration of protection will be necessary. It will also be of interest to determine whether cross-protection against other CVB serotypes can be achieved with this candidate vaccine (55). Although this recombinant virus induced both neutralizing and binding antibodies against the insert and vector in a mouse model, it is questionable if its use would be feasible in human applications due to the issues encountered with the insert instability. Moreover, even though the mouse studies showed no virus inhibition by preexisting anti-CVB3 antibodies, it needs to be evaluated if this proves true for other coxsackievirus serotypes.

Poxvirus—modified vaccinia Ankara. The family *Poxviridae* is divided into two subfamilies, *Chordopoxvirinae* and *Entomopoxvirinae*. Within the subfamily of *Chordopoxvirinae*, the genus *Orthopoxvirus*, which includes variola virus, the causative agent for smallpox, is interesting for human vaccine research. The most widely used candidate for new vaccine design is the vaccinia virus (VacV). This virus exhibits unique features that qualify it for use as an effective expression system and ideal recombinant vector. The large genome size of VacV (~190 kbp) allows the insertion of large amounts of foreign DNA (~25 to 30 kb) by homologous recombination, direct cloning, or bacterial artificial chromosome technology. This characteristic enables the design of vaccines against multiple pathogens within a single expression system. Vaccinia virus can also be applied via different routes (injection, oral) and induce long-lasting immunity. Many, so far unsuccessful, efforts to develop vaccines for complex diseases, such as tuberculosis

or malaria, that require a presentation of more than one antigen can potentially be overcome with a poxviral antigen presentation platform. The life cycle of VacV is fully accomplished in the cellular cytoplasm and does not require the integration of viral genetic material into the genome of the host. These attributes and the absence of a latent stage in the viral infection cycle are further advantages for vaccine design. The extensive investigation of VacV as a promising candidate for vaccine design has led to a number of modified VacV strains with higher safety profiles, more efficient expression systems, and higher immunogenicity in the host. Among other poxviruses (e.g., fowlpox, canarypox, or New York vaccinia virus), a most promising candidate for vaccine design is the attenuated vaccinia strain modified vaccinia Ankara (MVA). Relative to its parental strain (chorioallantoic vaccinia Ankara), MVA has lost 15% (~30,000 bp) of its genetic information at six major deletion sites during the attenuation process (57–60). These deletion sites have been shown to function as insertion sites for foreign genes into the MVA genome. Foreign genes can also be inserted upstream from endogenous poxviral promoters (*in situ*).

Fear of the next influenza pandemic has driven efforts toward the development of novel vaccines. As highly pathogenic avian influenza type H5N1 and heterologous influenza strains seem to be on the rise, Prabakaran and colleagues (61) developed a universal H5N1 vaccine candidate with broad coverage for pandemic preparedness. The HA genes of the A/Vietnam/1203/04, A/Indonesia/CDC669/06, and A/Anhui/01/05 (H5N1) strains were selected based on the neutralizing epitopes in HA covering most variants in the H5N1 clades. All three were inserted into deletion site 3 of MVAtor (Emergent BioSolutions, Gaithersburg, MD, USA), each under the control of a separate promoter (PsynI, PsynII, H5), resulting in the trivalent MVAtor-tri-HA. As a control, rMVAtor carrying only the A/Vietnam/1203/04 gene was constructed. Mice (BALB/c) immunized intramuscularly with a two-step protocol (day 0 and day 28) of 100 μl of 8×10^7 $TCID_{50}$ of MVAtor-tri-HA exhibited significant hemagglutination inhibition titers for the homologous viruses and the heterologous H5N1 clades, whereas the monovalent candidate induced only poor hemagglutination inhibition titers. A challenge experiment (ten 50% minimal lethal doses [MLD_{50}] intranasally) with a homologous clade 1 (RG-A/Vietnam/1203/04) and heterologous clade 7 (RG-A/chicken/Shanxi/2/06) H5N1 virus showed that the MVtor-tri-HA vaccine candidate conferred complete protection from weight loss and death in the immunized mice. In contrast, a previous experiment had shown that the monovalent counterpart conferred only 66% protection against the homologous H5N1 strain. Cross-clade immunity against 20 heterologous H5N1 clades was confirmed after a serological surveillance study in guinea pigs that were vaccinated with the trivalent vaccine candidate. The authors concluded that the robust and broadly neutralizing activity of their MVAtor-tri-HA vaccine candidate may also protect from yet unknown H5N1 strains. The poxviral vector seems to be an excellent delivery vehicle, as it has been proven safe and efficient in many monovalent recombinant vaccine candidates (61).

PROSPECTS, ADVANTAGES, AND CHALLENGES OF MULTIVALENT AND MULTIPATHOGEN VIRAL VECTOR VACCINES

With the number of vaccines growing and prevention (rather than treatment) being the most effective means of controlling virus infection, combination vaccines are becoming more important. Protection against several diseases with fewer injections while maintaining the efficacy and safety of single-component vaccines helps not only to reduce costs for health services and patients but also to simplify vaccine schedules (62). Despite all progress, some infectious diseases still claim millions of lives every year. For many of them, including malaria, leishmania, HIV, or tuberculosis, vaccine development has produced only suboptimal protection thus far. Viral vectors can potentially overcome this with their unique way of antigen presentation and capacity to express various transgenes at once. In countries with ineffective health services, combined vaccines would be easier to administer than their individual counterparts. Immunization coverage could be achieved more easily, with fewer visits to medical centers,

resulting in lower mortality, lower treatment costs, and lower levels of residual morbidity postinfection. Among new vaccine products, polyvalent and multipathogen viral vector vaccines hold great promise. Advantages of these recombinant vaccines include their ability to deliver multiple immunogens into the cells of the vaccinee, where they guarantee efficient expression. Conveniently, many of the viral vector shuttles present proteins to the immune system in the same way as that which occurs in a natural infection cycle and therefore ensure a potent induction of cellular and humoral immune responses (63). This route of antigen presentation holds the promise of long-lasting protection without numerous booster vaccinations. Many of the currently used vectors, such as modified vaccinia Ankara and measles virus vaccine, have been in use for years. They have accumulated significant safety and efficacy data through clinical and laboratory research through their use in prior vaccination applications. Progress in genetic engineering, recombinant DNA technology, and improved expression systems (promoter, terminator, enhancer, etc.) have advanced the field of recombinant vaccine design.

In using multipathogen or multivalent vaccines, special attention must be paid to the interaction of the various components with each other; potential interactions include antagonistic or synergistic effects or antigenic competition and/or epitope suppression, resulting in an inappropriate immune response (64). A common perception about vaccines containing more than one antigen is the overburdening of the immune system. Studies have shown that the immune system is responsive to more than 10 million antigens. With vaccines containing only a few specific antigens, an overload of a functional immune system is clearly extremely unlikely (65). Another challenge that needs to be addressed is the manufacturing and testing of new formulations. Each component of the vaccine must be assessed individually and in combination with standardized tests (stability, sterility, potency, efficacy, etc.) to ensure the consistency of the product (66). In addition, as more complex vaccines emerge, regulatory agencies will need to introduce new policies that provide guidelines for researchers, manufacturers, and practitioners regarding testing, licensing, documentation, information, and marketing of vaccines.

The enormous potential of viral vector vaccines drives the continuous development of novel expression vectors. Rhabdovirus- or influenza virus-based platforms are some of the latest to be suggested with the potential to express various antigens (67–70). The insertion of various transgenes into a vector is of course likely to lead to an impairment of virus replication. Revisiting traditional methods of viral gene expression, for example, by using conditional expression systems, such as Tet-on/Tet-off, may open the door for a new generation of improved viral vectors (71, 72).

Viral vectors have also been used in applications other than immunization against infectious disease, for example, as prophylactic or therapeutic cancer vaccines. Most recently, a study by Qiu and colleagues described a cytomegalovirus vector expressing modified tumor antigens. The vaccine candidate elicited tumor-specific T-cell responses, protecting mice from melanoma (73). Another promising study used a live attenuated poliovirus type 1 to vaccinate against glioblastoma multiforme (74). A valid concern in the use of viral vectors as vaccines is the possibility of the vaccinee developing vector immunity, resulting in reduced immunogenicity of the vaccine. Using a combination of different vectors for prime and booster vaccinations has been found to overcome this issue. Several examples in the literature describe the latter strategy, e.g., a recombinant adenovirus followed by an MVA booster regimen or a DNA prime followed by an adenoviral booster (75–78).

As discussed by Kreijtz and colleagues (79) in their excellent review on poxviral vectors, once regulatory challenges have been overcome, the implementation of the first human recombinant vaccine candidate into an existing vaccine schedule will lead to a major improvement in public health—conceivably sooner rather than later with the fast tracking of recombinant vaccines against Ebola virus disease (80–82). This would pave the way for taking multipathogen and multivalent vaccine candidates from the bench into clinical settings.

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