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Review

Live Attenuated Measles Vaccine as a Potential Multivalent Pediatric Vaccination Vector

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ABSTRACT

Live attenuated RNA viruses make highly efficient vaccines. Among them is the live attenuated measles virus (MV) vaccine that has been given to a very large number of children and has been shown to be highly efficacious and safe. MV vaccine induces a life-long immunity after a single injection or two low-dose injections. It is easily produced on a large scale in most countries and can be distributed at low cost. Reversion to pathogenicity has never been observed with this vaccine. For all of these characteristics, developing of MV vaccine vector as a multivalent vaccine to immunize children against both measles and other infectious agents such as human immunodeficiency virus (HIV), flaviviruses, or malaria might be very promising for worldwide use. As MV vaccine is inexpensive to produce, the generation of recombinant vaccines may remain affordable and attractive for the developing word. In this article, we describe the development of MV vector and present some recent data showing the capacity of recombinant MV vaccine to express various proteins from HIV and West Nile virus. In addition, the ability of recombinant MV to induce specific immune responses against these different pathogens are presented and discussed.

INTRODUCTION

MEASLES VACCINE, a live attenuated strain of measles virus (MV), is one of the most efficient and safest human vaccines available. It has been given to millions of children since the 1960s. Vaccination campaigns in developed countries have been very efficient in controlling measles. However, because of inadequate distribution of the vaccine in developing countries, measles still gives rise to 45 millions cases and 800,000 deaths among children every year, thus leading the World Health Organization (WHO) to strengthen a worldwide mass vaccination program for the next 10–20 years (8). Taking advantage of these campaigns, live attenuated MV vaccine could provide safe and efficient bivalent pediatric vaccines to immunize children against measles and at the same time against other infectious diseases in the developing world.

Measles virus belongs to the genus *Morbillivirus* in the family *Paramyxoviridae*. It is an enveloped virus with a 15,894-nucleotide long non-segmented RNA genome of negative polarity. In 1954, Enders and Peebles inoculated primary human kidney cells with the blood of David Edmonston, a child with measles (19), and the resulting Edmonston strain of MV was subsequently adapted to growth in a variety of cell lines. Adaptation to chicken

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embryos, chick embryo fibroblasts (CEF), and/or dog kidney cells and human diploid cells produced the attenuated Edmonston A and B, Zagreb (EZ), and AIK-C seeds (24,26,44). Edmonston-B was licensed in 1963 as the first MV vaccine. However, because it was reactogenic it was abandoned in 1975. Further passages of Edmonston A and B on chick embryo fibroblasts produced the more attenuated Schwarz, and Moraten vaccines (46). Although all attenuated strains are highly homologous, Moraten and Schwarz strain sequences have been shown to be identical (39,40). Today, the Schwarz/Moraten, AIK-C, and EZ vaccines are commonly used. MV vaccine induces a life-long immunity after a single or two low-dose injections (24,26). Persistence of antibodies and CD8 cells has been shown for as long as 25 years after vaccination (37). MV vaccine is easily produced on a large scale in most countries and can be distributed at low cost. Because the attenuation of MV genome results from an advantageous combination of numerous mutations, the vaccine is very stable and reversion to pathogenicity has never been observed (26). With regard to safety, MV replicates exclusively in the cytoplasm, ruling out the possibility of integration into host DNA. These characteristics make live attenuated MV vaccine an attractive candidate for use as a multivalent vaccination vector. By taking advantage of the existing technology to produce and distribute large quantities of MV vaccine and by exploiting its capacity to induce efficient immune responses, live attenuated MV could be used as a multivalent pediatric vaccination vector to mass-immunize children and adolescents against both measles and other infectious diseases.

To this end, a reverse genetics system for MV was established allowing the rescue of infectious MV from cloned cDNA (43). Multiple cloning sites were introduced into the MV genome that allows the addition of foreign genes and their continuous expression by the progeny. Initially, several marker genes (chloramphenicol acetyltransferase [CAT], Green fluorescent protein [GFP], and β-galactosidase [B-gal]) were expressed. Then, several genes or combinations of genes from hepatitis B virus (HBV), simian or human immunodeficiency viruses (SIV, HIV), mumps virus (MuV), West Nile virus (WNV), and human IL12 were inserted at different loci in the MV genome (15,31,42,47–49,52). The corresponding viruses expressed these genes in a stable manner.

GENE EXPRESSION BY MV VECTOR

The MV vectors carrying either reporter genes at different sites or a combination of reporter genes are not only useful for developing MV vector but also find various direct applications. CAT expressing MV has been useful for detection of MV spread from the site of inoculation (lung) to peripheral organs (kidney, spleen) in transgenic mice (34). β-gal–expressing MV has also proved invaluable for the straightforward monitoring of MV in the brain of intracranially infected mice. The GFP reporter transgene has been inserted in one of three genome positions (Fig. 1). As expected, the strongest expression was seen from the site upstream of the nucleoprotein (N) reading frame, the lowest from the site downstream of the hemagglutinin (H) reading frame. These MV recombinants allowed the direct time-lapse monitoring of details of MV spread in various cell cultures. Curiously, it has been found that MV vectors infect single cells around syncytia well before fusion ensues, in contrast to mutants deficient in matrix protein (M) (10), or undergo alterations in their glycoprotein tails, which lead to direct and much more rapid cell fusion (11). A "triporter" MV vector was constructed, carrying all three reporter genes in the three defined genomic positions shown in Fig. 1. This virus contains more than 5000 exogenous nucleotides, elongating the MV genome length by more than 30%. In addition, double recombinants expressing SIV or HIV Env and Gag proteins cloned in positions 2 and 3 respectively, with approximately equal insert size, were rescued easily and grew to relatively similar titers as the parental vaccine (A. Zuniga et al., manuscript in preparation). Foreign viral antigens from SIV or HIV proteins did not incorporate into the MV envelope, indicating suitability for expression without risk of affecting the virus tropism.

Stability of gene expression. The heavily loaded viruses multiply slightly more slowly than standard MV but are produced at similar titers. Even more gratifying was the observation that after 12 serial passages encompassing a total amplification of 10–20, more than 96% of the syncytia formed stained positive for all three reporter genes. The remarkable genetic stability of added, expressed ORFs, which has been observed also in other members of the Mononegavirales (45), is difficult to understand in view of the fact that several plus-strand RNA viruses with added genetic RNA segments often partially or fully delete their inserts even after a few rounds of infection in cell culture. The stability is likely due to the fact that there is little constraint on genome size for pleomorphic viruses with a helical nucleocapsid. Other factors such as the viral polymerase complex may play an internal proof-reading control.

Immune response to recombinant MV. To explore the immunogenicity of expressed heterologous antigens, transgenic mice were immunized with MV expressing B-gal (35), and several HBV (48), HIV (31), or WNV (15)

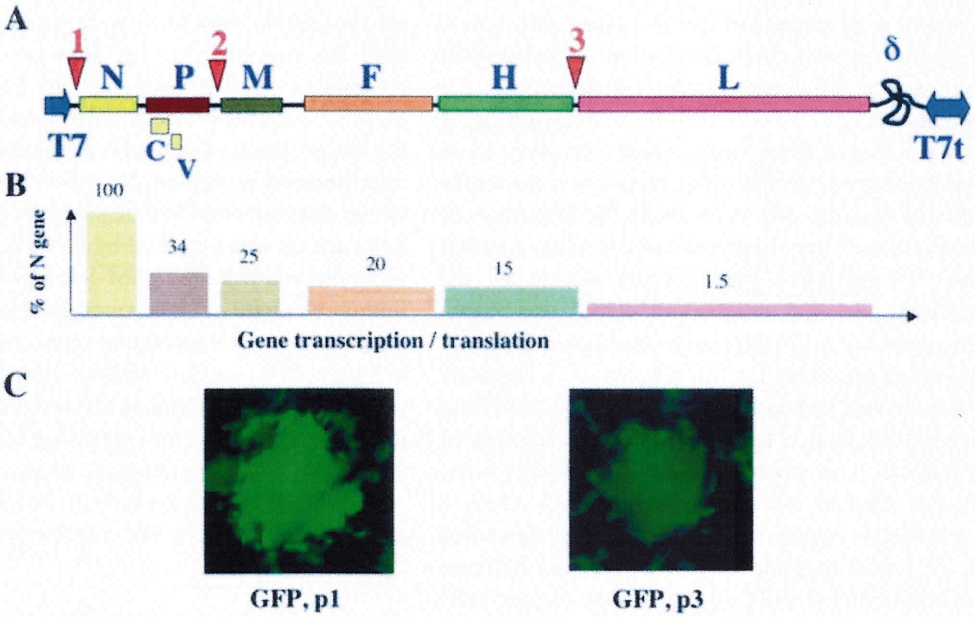

FIG. 1. (A) Schematic presentation featuring cloning positions of foreign transgenes into the measles virus (MV) genome. N = nucleoprotein; P = phosphoprotein; M = matrix protein; F = fusion protein; H = hemagglutinin; L = large polymerase protein; T7 = T7RNA pol promoter; T7t = T7 RNA pol terminator; δ = hepatitis delta virus (HDV) genomic sense ribozyme. Total number of nucleotides of the MV genome: 15,894. (B) Organization of MV gene expression. N is the mostly expressed gene and L is the least expressed. MV genes are thus expressed in a gradient fashion. ATUs were introduced at three distinct positions, indicated by arrows, to obtain high or low expression of proteins. The strength of foreign transgene expression is thus dependent on the position of its insertion. (C) Variation of expression of Green Fluorescence Protein (GFP) by recombinant MV. GFP is expressed from either position 1 or position 3.

antigens. Interestingly, because MV efficiently infects professional antigen presenting cells (macrophages and particularly dendritic cells), MV-based vectors deliver their foreign cargo directly to the most efficient antigen presenting cells. The MV vector expressing HBV surface and core antigens was tested by both intranasal and intraperitoneal inoculation. High levels of antibodies directed against MV, quantified both by enzyme-linked immunoassay and neutralization tests, were detected, and reasonable amounts of antibodies to HBV s antigen were detected (48). Mice immunized with MV-HIV recombinants developed strong and enduring humoral and cellular immune responses against the HIV antigens (31). An MV-WNV recombinant expressing the secreted form of WNV E glycoprotein induced strong humoral responses that protected immunocompromised mice from a lethal WNV challenge (15). The responses generated were caused by replication of recombinant viruses, as ultraviolet-inactivated viruses led only to background levels of antibodies. In addition, envelope exchanges between MV and vesicular stomatitis virus (VSV) have shown viable and immunogenic chimeric viruses that protected immunocompromised mice from lethal doses of VSV (21,49).

CLONING OF CLINICALLY CERTIFIED MV VACCINES

We recently developed MV vectors based on the safest and most widely used MV vaccines, the Schwarz and the Edmonston Zagreb (EZ) strains. We showed that the molecularly cloned Schwarz virus was as immunogenic as the parental vaccine in primates and mice susceptible to MV infection (14). In the present paper, we summarize results recently obtained on animal immunization and protection with recombinant MV Schwarz vectors expressing either HIV or WNV proteins.

A recombinant MV vaccine intended to immunize children against measles and at the same time against another infectious disease must be derived from an approved and efficient strain of MV vaccine. Therefore, two clinically approved strains of MV vaccines were cloned for use as vectors: (1) the Schwarz vaccine strain, constituent of several widely used measles vaccines, Attenuavax (Merck and Co. Inc., Whitehouse Station, NJ), Rouvax (Aventis Pasteur, Marcy l'Etoile, France), Priorix (GSK Biological, Rixensart, Belgium), and of the combined vaccine MMR (7); and (2) the Edmonston Zagreb vaccine strain (Berna Biotech Ltd, Bern, Serum Institute of

India), widely used in parts of Europe, Asia, and Africa. The EZ strain was also clinically used as an aerosolized vaccine in South Africa and Mexico (4). Infectious cDNAs corresponding to the anti-genome of Schwarz or EZ (Fig. 2) were cloned from viral particles prepared from industrial batches of vaccine using procedures optimized for fidelity of cloning (14). As a result, the sequences of the cloned genomes were identical to those of the parental genomes. To maximize yield during rescue of the Schwarz strain, the viral antigenomic cDNA was placed under the control of a T7 RNA polymerase promoter with the GGG motif necessary for full efficiency. A hammerhead ribozyme was inserted between this GGG motif and the first viral nucleotide to allow the exact cleavage of the viral RNA. The resulting plasmid pTM-MVSchw (Fig. 2) was used to rescue the Schwarz MV using a helper-cell-based rescue system previously described (38,43). To avoid adapting both Schwarz and EZ vaccines to non-certified cells during rescue, helper cells transfected with the engineered cDNAs were co-cultivated with chicken embryo fibroblasts for Schwarz and MRC5 for EZ, the cells on which these vaccines are usually grown and produced. The rescued viruses were passaged several times and their genomes sequenced. No mutation was found when the sequences were compared to that of the original viruses. Moreover, the growth kinetics and the yields of the rescued viruses were identical to the original viruses. Both Schwarz and EZ viruses could also be rescued after co-cultivation of transfected helper cells with Vero cells, which are very permissive to MV.

The pTM-MVSchw plasmid was engineered for the expression of foreign genes by the introduction of additional transcription units (ATU) at different positions of the genome. This ATU is a multiple cloning site cassette inserted into a copy of the N-P intergenic region of MV genome (42). This region contains the cis-acting sequences necessary for the transcription of the MV-P gene. The Green Fluorescent Protein (GFP) sequence was inserted in the cassette. The ATU was introduced into pTM-MVSchw in two different positions (between the P and M genes and between the H and L genes) (Fig. 2). Similarly, the cloning of EZ followed procedures published earlier (43). The total number of antigenomic nucleotides was kept as a multiple of 6 (9). Recombinant viruses were rescued from the resulting plasmids and used to infect CEF, Vero, or MRC5 cells. The GFP transgene was expressed in all infected cells, thus showing that this Schwarz MV functions as a vector.

The immunogenicity of the virus rescued from pTM-MVSchw plasmid and passaged two times on CEF or Vero cells was for evaluated in cynomolgus macaques and compared to that of the industrial Schwarz vaccine. These macaques are sensitive to MV infection or vaccination (29,51). Monkeys were injected sub-cutaneously with the standard dose of Schwarz MV vaccine from Aventis or Schwarz MV rescued from pTM-MVSchw plasmid. All of the vaccinated macaques became positive for the presence of anti-MV antibodies and specific cellular immune responses. No difference was observed between the Schwarz MV rescued from plasmid and the Schwarz vaccine prepared by Aventis, indicating that the virus rescued from the pTM-Schw plasmid had the same immunogenicity in macaques as the parental Schwarz vaccine (14). This molecular clone allows producing the Schwarz MV vaccine without having to rely on seed stocks. With its additional transcriptions units, it is possible to use it as a vector expressing foreign proteins. This vector, which is immunogenic in macaques, allows constructing recombinant vaccines based on an approved, widely used, efficient MV vaccine strain.

RECOMBINANT MV EXPRESSING HIV-1 ENVELOPE GLYCOPROTEINS

The vast majority of the 40 million individuals currently infected by HIV are living in developing countries (UNAIDS/WHO report, 2002). In these areas, mother-to-child transmission, including through breast-feeding, accounts for one half million infections every year, and most cases of sexual transmission occur in individuals less than 20 years of age. Therefore, developing a preventive pediatric HIV vaccine is a major goal in the fight against AIDS. Such a vaccine must be easy to produce on a large scale and at low cost in developing countries. It must be safe and able to induce protective immunity after one or two injections. By taking advantage of the existing technology to produce and distribute large quantities of MV vaccine, live attenuated MV-HIV could be used as a bivalent AIDS vaccination vector to mass-immunize children and adolescents against both measles and AIDS.

To explore this possibility, we produced recombinant MVs expressing different forms of HIV envelope glycoprotein, and we evaluated their immunogenicity in mice and macaques (31). To favor the induction of cross-reactive neutralizing antibodies, we deleted the hypervariable V1, V2, or V3 loops of the $HIV_{89.6}$ gp160 (membrane-anchored) and gp140 (soluble) proteins to eliminate these "immunological decoys" and to expose more conserved epitopes (2,5,28). The sequences coding for these proteins were inserted into the MV vector. Recombinant viruses were produced and the expression of the HIV-Env protein was analyzed in infected-cell lysates (Fig. 3). The different native and mutant $Env_{HIV89.6}$ gp140 and gp160 were efficiently expressed and correctly matured. After five passages of the recombinant MVs on

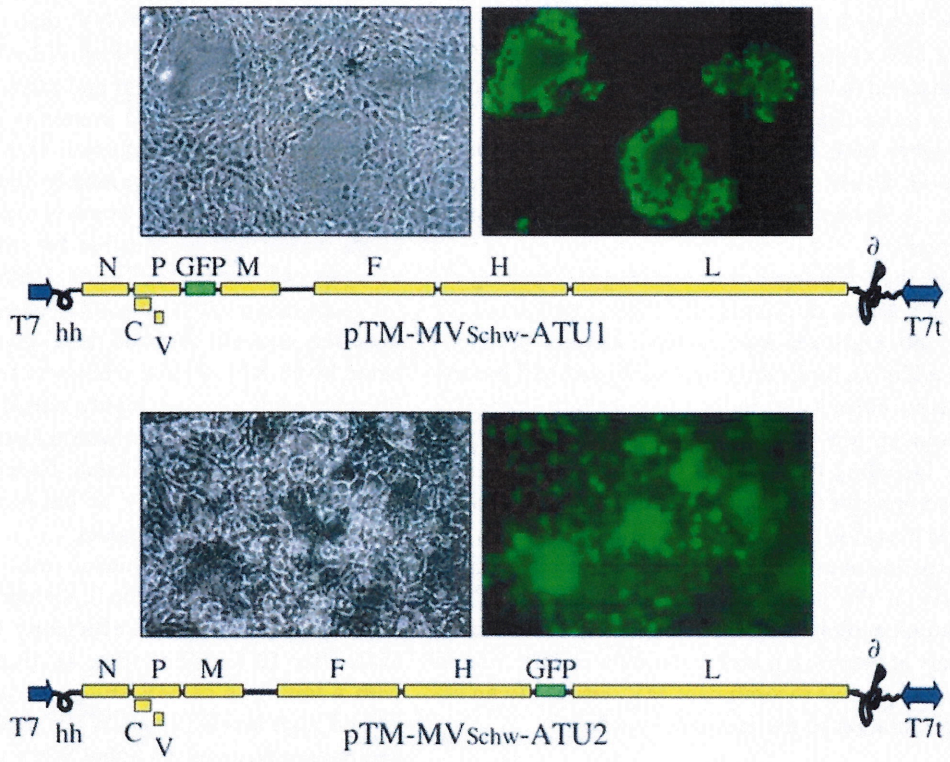

FIG. 2. The Schwarz measles virus (MV) vector. All designations are as in Fig. 1, except a hammer-head (hh) ribozyme was added. Plasmid = pTM. The GFP gene is inserted in additional transcription units as indicated. The expression of GFP in infected Vero cells is shown in the lower part.

Vero cells, transgenes expression was similar, confirming the stability of expression in this system. We shown that the growth of MV-$Env_{HIV89.6}$ recombinant viruses was only slightly delayed compared to that of standard MV, and that the final yield of recombinant viruses was comparable to that of standard MV.

The immunogenicity of MV-$Env_{HIV89.6}$ viruses was tested in genetically modified mice expressing the human CD46 MV receptor and lacking the type I interferon receptor (31). Our results show that MV-HIV recombinants induced high titers of antibodies against both MV and the HIV Env constructs. A high level of MV- and HIV-specific $CD8^+$ and $CD4^+$ cells was also induced. As much as 7% of the total $CD8^+$ T-cells and 4% of the total $CD4^+$ T-cells were MV specific and 1.7% of the total $CD8^+$ T-cells and 0.9% of the total $CD4^+$ T-cells were HIV specific. Hence, MV-$Env_{HIV89.6}$ recombinant viruses are genetically stable; they express the HIV Env protein at high levels; and they induce humoral and cellular immune responses against both MV and HIV Env.

Most importantly, we demonstrated that pre-existing antibodies to the vector did not impair the immunogenic potential of the recombinant virus that was able to induce anti-HIV antibodies in pre-vaccinated mice and macaques, provided that two injections were administered (31).

We also found that a single injection of MV-HIVEnv induced anti-HIV antibodies that were able to neutralize the homologous SHIV89.6p virus as well as several heterologous clade A and clade B HIV-1 primary isolates (31). The hypervariable loops deletion increased the level of antibodies neutralizing homologous SHIV89.6p as well as heterologous primary HIV-1 isolates (Fig. 3) This observation suggests that removing the hypervariable regions may have exposed some immunological elements conserved among several HIV-1 isolates. Hence, hypervariable loops deletion broadens humoral immune responses (2,28,50). The gp160 expressed by MV appears to be a better antigenic mimic of the envelope glycoprotein trimeric complex present on HIV virions than is the gp140.

Although the correlates of immune protection against HIV-1 infection are still not completely understood, a preventive immunization ideally should induce specific T lymphocytes as well as antibodies that neutralize primary isolates. Our results indicate that this might be possible using an already existing live MV vaccine as a vector for expressing HIV proteins. To broaden the immune

responses induced by the recombinant virus, we constructed a MV expressing both the gp140 and the Gag proteins inserted in the two ATU of the vector. This virus, containing more than 4,000 additional nucleotides, expressed stably both the proteins at high levels and induced good levels of cellular immune responses in macaques. A Nef-expressing MV was also shown to be immunogenic.

What are the advantages of MV among the existing viral vector systems developed for HIV immunization? Practical and logistical aspects have already been exposed. In addition, replicating live attenuated MV has the advantageous capacity to induce long-lasting immunity (26). Moreover, MV and HIV have several properties in common, including the fact that they both infect monocytes, macrophages and dendritic cells (20,27). Therefore, an MV vector that targets the HIV proteins in the same compartment as HIV itself may provide advantages, particularly in the induction of "danger signals" (33). Good immunogenicity in our system likely results from good levels of expression and maturation of HIV Env in cells infected by live recombinant MV, leading to an efficient presentation to the immune system.

PREVENTION OF HETEROLOGOUS VIRAL DISEASE USING MV VECTOR

In a recent study, we evaluated whether immunization based on MV vaccine can protect against a heterologous, medically important pathogen such as West Nile Virus. WNV is a single-stranded RNA virus of the *Flaviviridae* family, genus flavivirus, within the Japanese encephalitis antigenic complex (6,41). Since its introduction into the United States in 1999, WNV infection has been one of the most serious mosquito-borne disease in the Western Hemisphere (41). Outbreaks of WNV in recent years have coincided with the emergence of the highly virulent variant Isr98/NY99 of WNV that circulates in North America and the Middle East (12,13,30). WNV infects the central nervous system and causes severe neurological disease (36). Humoral immunity plays a critical role in clearance of WNV infection (1,3,17,25). Anti-WNV neutralizing antibodies are mainly directed against the envelope (E) glycoprotein, which is exposed on the surface of the virion and responsible for virus attachment and virus-specific membrane fusion. Based on these findings, we generated a recombinant Schwarz MV vector to produce the secreted form of the E-glycoprotein of WNV strain IS-98-ST1, a close relative to Isr98/NY99 (2,13,2). Because adult mice are highly sensitive to a low-dose of IS-98-ST1 WNV after peripheral inoculation (16,32) and develop a neuroinvasive lethal disease similar to human disease (12), the efficacy of the recombinant MV was evaluated in the mouse model.

We produced a recombinant virus, MV-sE_{WNV}, which expresses high levels of the E glycoprotein of WNV. The WNV protein is secreted efficiently from infected cells, even after 10 passages (Fig. 4). Immunization of mice with a low dose of MV-sE_{WNV} resulted in the production of high levels of anti-WNV neutralizing antibodies and protection against a lethal WNV challenge (15). Passive administration of low amounts of antisera from immunized mice prevented WNV encephalitis in BALB/c mice challenged with a high dose of WNV. Moreover, we demonstrated that mice were protected as soon as 8 days after a single injection with a low-dose of recombinant vaccine and remained protected 6 months later. This study demonstrates for the first time that recombinant live-attenuated MV is effective at preventing a heterologous viral disease.

With its capacity to induce a strong, long-lasting protective immunity, the live-attenuated MV expressing the E-glycoprotein could potentially serve as medical vaccine for simultaneous prevention of measles and WNV. Because of cross-species transmission, it is feared that

FIG. 3. Expression of human immunodeficiency virus (HIV-1) Env glycoproteins by measles virus (MV) vector and induction of anti-HIV neutralizing antibodies in immunized mice. (**A**) expression of HIV-$1_{89.6}$ gp160, gp140, mutants with deletion of hypervariable regions, and of MV nucleoprotein (N) detected in lysates of Vero cells infected by MV-Env_{HIV} viruses (P2 and P5 are successive passages of the recombinant viruses). HIV proteins are probed with mouse monoclonal anti-HIV gp120, and MV N protein with a monoclonal anti-MV N. (**B**) Immunofluorescence staining of syncytia induced in Vero cells infected by MV-Env_{HIV} viruses (staining with anti-HIV-1 Mab 2g12). (**C**) *In vitro* neutralization of homologous SHIV89.6 and heterologous HIV-1 BX08 primary isolate by sera from MV-$Env_{HIV89.6}$ immunized mice (collected 28 days after immunization). Mutants with hypervariable region deletions induce higher titer of neutralizing antibodies.

FIG. 4. Expression of West Nile Virus (WNV) sE glycoprotein by MVSchw-sE_{WNV} recombinant measles virus (MV) in Vero cells (see text for abbreviations and explanation). (**A**) Schematic diagram of MVSchw-sE_{WNV}. (**B**) Growth curves of MVSchw-sE_{WNV} in Vero cells (MVSchw, open box and MVSchw-sE_{WNV}, black box). (**C**) Immunofluorescence staining of WNV sE glycoprotein in syncitia of MVSchw-sE_{WNV}-infected Vero cells (staining with anti-WNV HMAF, A-B cells were permeabilized with trition, C-D cells were not permeabilized). (**D**) Radioimmunoprecipitation assay showing the release of sE_{WNV} from MVSchw-sE_{WNV}-infected Vero cells (immunoprecipitation with specific anti-MV and anti-WNV polyclonal antibodies).

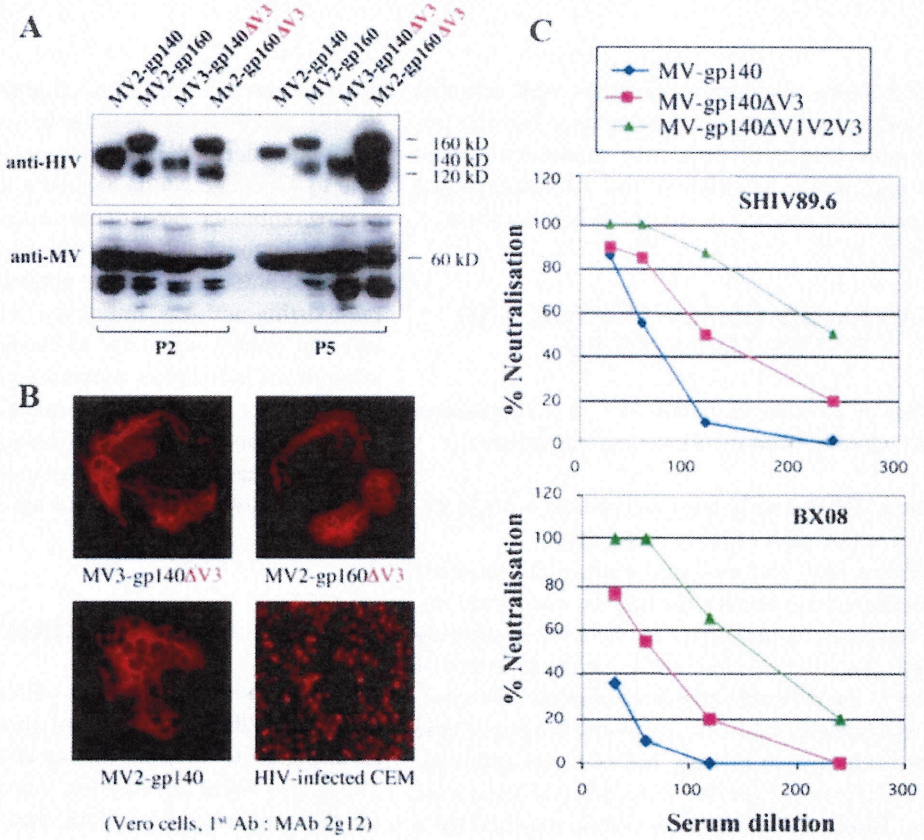

FIG. 3.

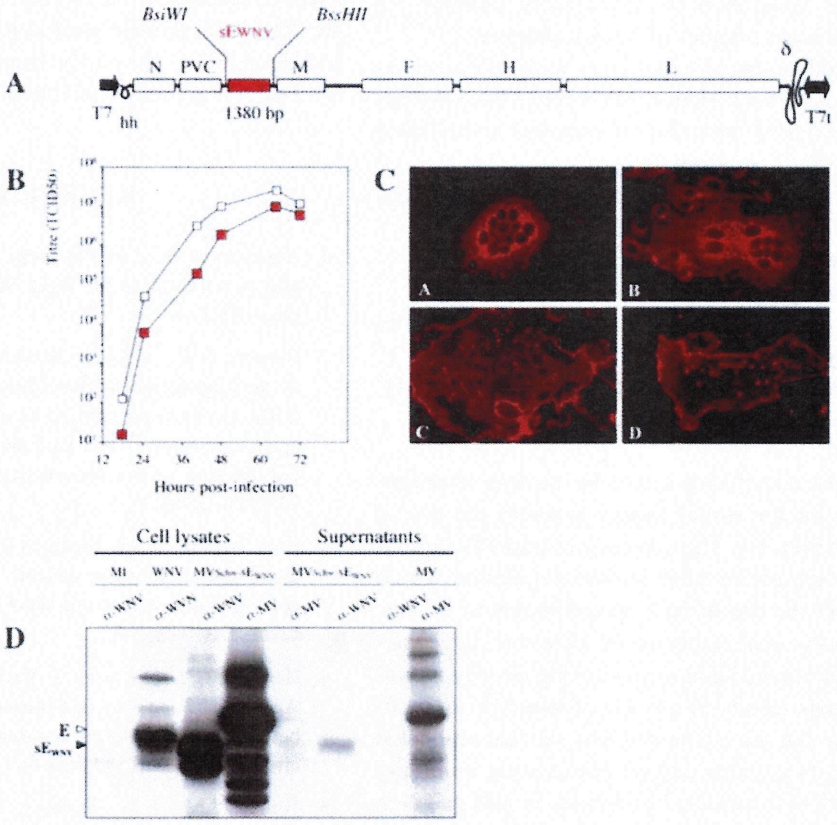

FIG. 4.

WNV will become a recurrent zoonosis with repeated seasonal outbreaks in humans. MV-sE_{WNV} has the potential to raise long-term protective immunity against both MV and WNV in children and adolescents that might be naturally boosted in case of WNV outbreak.

ADVANTAGES OF MV VECTOR AND ISSUES TO RESOLVE

A number of advantages to use MV as a vaccination vector have already been mentioned in this article:

- Attenuated MV is widely used worldwide, with an excellent record of high efficacy and safety.
- Recombinant MV vectors could replace the standard MV vaccine used routinely for infants worldwide under the auspices of the WHO in the MV eradication campaign. It should be mentioned that for a variety of reasons it is questionable whether complete MV eradication will be achievable. In any event, the target time for eradication, which initially has been set optimistically to 2010 is now expected to be delayed for at least 10 years. Thus, MV vaccination will be required for a long period to come and possibly forever.
- MV encodes only a small number of proteins, thus avoiding the generation of unnecessary immune responses to a large number of vector antigens.
- There is good practical experience with MV vaccine application as aerosol. Hence, MV vectors have the potential for efficient induction of mucosal neutralizing immunity against vectored antigens.
- Large numbers of vaccination doses can be produced easily and very economically.
- No viral genome integration is possible.
- Genetic stability is excellent.
- No recombination has been demonstrated within paramyxoviruses.
- No persistence and no dissemination of attenuated MV strains have been demonstrated.

However, these advantages could be useless if preimmunity to the vector would impair severely the use of MV vectors in humans. The presence of anti-MV immunity in nearly the entire adult human population would seem to restrict the use of MV recombinants to infants, an already worthy goal in any event. However, large vaccination studies showed that revaccinating already immunized individuals results in a boost of anti-MV antibodies, suggesting that the attenuated live vaccine replicated and expressed its proteins despite pre-existing immunity (18). Studies of cell-mediated immunity in infants given measles vaccine during the first year of life show that the presence of maternal anti-measles antibodies does not prevent the induction of anti-measles cellular responses and that responses can be very efficiently boosted (22,23). Under such circumstances, one might hope to be able to vaccinate adults against a foreign antigen with a MV recombinant. Indeed, our experiments with MV-HIV viruses demonstrate, at least in mice and macaques, that anti-HIV antibodies can be obtained in the presence of pre-existing anti-MV immunity (31). Whether MV vectors can induce immunity to carrier proteins in already immunized individuals remains to be evaluated. Phase I clinical trials should be performed to evaluate the safety of MV vectors. Such trials should identify a suitable dose of recombinant MV and to demonstrate an acceptable reactogenicity profile and the absence of *in vivo* virus shedding.

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