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Attenuated Mengo virus as a vector for immunogenic human immunodeficiency virus type 1 glycoprotein 120

(vaccine)

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ABSTRACT **Introduction of a sequence encoding 147 amino acids from human immunodefiency virus type I (HIV-1) strain MN glycoprotein gp120 into the RNA genome of the stably attenuated Mengo virus strain vM16 yielded an infectious recombinant virus, vMLN450, which expressed the heterologous HIV-1 sequence along with the normal Mengo virus proteins. The HIV-1 gp120 sequence, fused to the amino terminus of the short, nonstructural Mengo virus leader polypeptide was recognized by a gp120 V3 loop-specific monoclonal antibody. When inoculated into mice, recombinant virus vMLN450 elicited a high-titer anti-HIV-1 antibody response as well as an HIV-1_{MN}-specific cytotoxic cellular immune response. An anti-HIV-1 antibody response could also be detected in cynomolgus monkeys after a single immunization. We propose that attenuated Mengo virus can serve as an effective expression vector in cell systems and various animal species and offers another approach to the development of new, live recombinant vaccines.**

In view of the complexity of the immune response to human immunodeficiency virus type 1 (HIV-1) (1), it is necessary that effective vaccines induce a strong humoral immune response and a long-lived cellular immunity. Such responses are typically obtained with live virus vaccines. Ideally, viral vectors to be used as live recombinant vaccines should (*i*) carry stable attenuating mutations, (*ii*) be unable to integrate into the genome of the host, (*iii*) show minimum propensity for persistence after inoculation, (*iv*) be easy to administer prophylactically, and (*v*) efficiently express the heterologous sequences in an immunologically relevant manner.

Picornaviruses such as poliovirus, rhinovirus, or Mengo virus are attractive models for the development of recombinant vaccines because their RNA genomes are expressed exclusively in the cytoplasm of infected cells. We have attempted to develop Mengo virus as another viral vector. Mengo virus is a cardiovirus that shares the same serotype as the encephalomyocarditis virus, and it can replicate in a wide range of animal species, including primates (2–5). Genetic engineering has shown that the pathogenic potential of Mengo virus is controlled by a homopolymeric poly(C) tract ($C_{50}UC_{10}$) within the 5′ noncoding region of the genome (6, 7). Truncation or deletion of the poly(C) tract leads to stably attenuated Mengo virus strains that can be propagated with ease in cell culture and are highly resistant to reversion. The short poly(C) strains, such as the vM16 strain [poly(C) tract of $C_{13}UC_{10}$], have lost pathogenic potential for BALB/c and Swiss mice (7), domestic pigs, baboons, and rhesus macaques (A.P., S.v.d.W., M.G., unpublished work) and induce a lifelong protective immunity in mice and pigs against virulent Mengo virus or encephalomyocarditis virus infection (A.P., unpublished work).

Like all picornaviruses, the Mengo virus genome encodes a large polyprotein that is cleaved proteolytically into a series of mature structural and nonstructural proteins (3). Unlike entero- and rhinoviruses, the P1 capsid region of cardio- and aphthoviruses is preceded by a leader polypeptide (L polypeptide). The Mengo virus L polypeptide is 67 amino acids in length. Its functional relevance to the virus is unknown, as it is not a protease like the L protein in aphthoviruses. Release of L polypeptide from the polyprotein requires proteolysis by viral protease 3C, an enzyme encoded downstream in the viral genome. To determine whether attenuated Mengo virus could be used as a viral vector and have potential to serve as a live recombinant vaccine, we engineered a segment encoding the V3–C4 domains of HIV-1 strain MN glycoprotein 120 (gp120) into the region of the stably attenuated Mengo virus strain vM16 genome that encodes the L polypeptide. The resulting recombinant virus expressed the gp120–L polypeptide fusion protein along with the normal Mengo virus proteins and elicited a strong humoral, as well as cellular, immune response to HIV-1 in immunized animals.

MATERIALS AND METHODS

Construction of Recombinant Virus vMLN450. All DNA manipulations were done according to standard procedures (8). Subclone pMRA1 was generated by deletion of the 5.8-kb *Sph* I–*Sph* I fragment (plasmid base 1928–7775) from plasmid pM16, which contains the vM16 Mengo virus cDNA downstream of the T7 promoter (6). HIV-1_{MN} gp120-specific DNA was amplified by PCR from plasmid pTG5156 (Transgene, Strasbourg, France), provided by M. P. Kieny, using oligonucleotides 5′-VCN (ATATGTTGACCATGGAACAAATTAATTGTACAAGACCC) and 3′-VCN (TAATCCATGGCGGTCAACGTGGGTGCTACTCCTAATGG). The PCR fragment was first cloned into subclone pMRA1 at the *Nco* I site (viral base 729), resulting in plasmid pMRA2. Full-length cDNA was reestablished by transfer of the 5.8-kb fragment of plasmid pM16 into the *Sph* I site of plasmid pMRA2, resulting in plasmid pMRA3. Correct orientation of cloned sequences was determined by PCR, and plasmids were amplified in *Escherichia coli* strain DH5α (Promega).

Infectious RNA transcripts derived from plasmid pMRA3 were prepared by using T7 RNA polymerase and transfected into HeLa cells as described (6), resulting in recombinant Mengo virus vMLN450. Stocks of viruses vM16 and vMLN450 were produced by passage on HeLa cells, titrated as described (9), and analyzed by reverse transcription-PCR for the presence of the inserted HIV-1 sequence using Mengo virus-specific oligonucleotides, 5′194 (TAGGCCGCGGAATAAGGCCGGTGTGC) and 3′1094 (GGAGCATGTTCGAGAAAGCATTGAC).

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Abbreviations: L polypeptide, leader polypeptide; CTL, cytotoxic T lymphocyte; pfu, plaque-forming unit(s); gp, glycoprotein.

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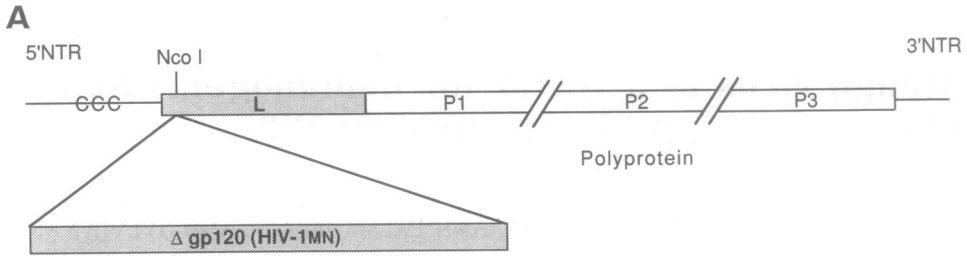

B

Δgp120-L Fusion Protein (25.5 kD, 220 aa)

MATTMEQINC TRPNYNKRKR IHIGPGRAFY TTKNIIGTIR QAHCNISRAK WNDTLRQIVS
(299)
KLKEQFKNKT IVFNQSSGGD PEIVMHSFNC GGEFFYCNTS PLFNSTWNGN NTWNNTTGSN
NNITLQCKIK QIINMWQEVG KAMYAPPIEG QIRWLTAMEQ EICAHSMTFE ECPKCSALQY
(445)
RNGFYLLKYD EEWYPEESLT DGEDDVFDPD LDMEVVFETQ

FIG. 1. Construction of recombinant virus vMLN450. (*A*) The cDNA sequence encoding amino acids 299–445 of HIV-1_{MN} gp120 was inserted between amino acids 5 and 6 of the L polypeptide in pM16 cDNA at the beginning of the viral polyprotein open reading frame. (*B*) Sequence of the resulting fusion protein Δgp120–L polypeptide. Leader amino acids are represented in normal type, and gp120 amino acids are in boldface type. Additional residues encoded by the DNA linkers are underlined. kD, kDa; NTR, nontranslated region.

Immunizations and ELISA Tests. Ten-week-old BALB/c mice were immunized twice i.p. with 10^6 plaque-forming units (pfu) of recombinant virus vMLN450 or 10^6 pfu of virus vM16 in phosphate-buffered saline (PBS) on days 0 and 56. Control animals received PBS alone. Adult cynomolgus monkeys were immunized i.m. with 10^6 pfu of virus vMLN450 or vM16 in PBS. ELISA plates (Nunc Maxisorb) were coated with 50 ng per well of purified recombinant gp160LAI or recombinant gp120MN fused to gp41LAI (gp160MN–LAI; Transgene), incubated with serial dilutions of sera, followed by horseradish peroxidase-conjugated sheep anti-mouse-IgG (H+L) antibody (Diagnostics Pasteur, Marnes la Coquette, France) or rabbit anti-monkey-IgG (H+L) antibody (Nordic, Tilburg, The Netherlands). Commercial ELISA tests (ELAVIA; Diagnostics Pasteur) were done by following the manufacturer's protocol. HIV-1_{MN} neutralization assays were done as described (10) by using 3.5×10^5 MT4 cells. Syncytia formation was monitored between days 6 and 10.

Cytotoxicity Assay. BALB/c mice were immunized i.p. on days 0 and 21 with 10^6 pfu of virus vM16 or vMLN450. Spleen cells from three mice per lot were recovered 10 days later, pooled and restimulated *in vitro* for 7 days with P18-MN peptide (11), and then stimulated for 5 days with P18-MN peptide in the presence of 5% ConA supernatant-containing medium as a source of growth factor. Cytolytic activity of stimulated splenocytes was determined by a 5-hr ^{51}Cr-release assay. Target cells were peptide-pulsed ^{51}Cr-labeled P815 tumor cells. Percentage of specific ^{51}Cr release was calculated as [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. Spontaneous release was <20% of maximal release obtained by incubation with 1% Triton X-100.

RESULTS

Construction of Recombinant HIV-1/Mengo Virus vMLN450. A 459-nt cDNA segment coding for amino acids 299–445 from the gp120 of HIV-1_{MN} was generated by PCR and inserted at viral position 729 of vM16 cDNA. The resulting plasmid, pMRA3, carried a short poly(C)-tract Mengo virus cDNA that encoded 147 amino acids from the V3–C3–V4–C4 domains of the gp120 of HIV-1_{MN}, fused in-frame between amino acids 5 and 6 of the L polypeptide of Mengo virus (Fig. 1). RNA transcripts were prepared from plasmid pMRA3 and transfected into HeLa cells. The cells showed clear cytopathic effect ≈72 hr after transfection, as compared with 48 hr for cells transfected with plasmid pM16 RNA. Viable virus was harvested and designated vMLN450. Plaque size of the recombinant virus was ≈65% that of vM16 (Fig. 2). When passaged in HeLa cells, vMLN450 grew to titers ≈10% those of vM16, and the insert sequence was stably retained in the vMLN450 genome for at least four passages, as assessed by reverse transcription-PCR analysis of the viral genome (data not shown).

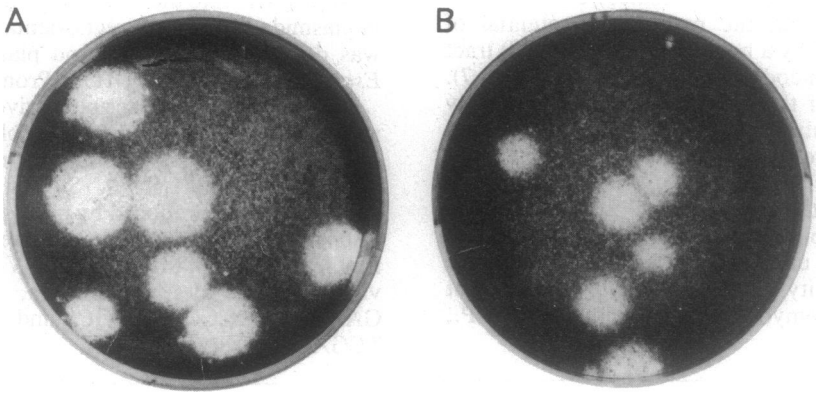

FIG. 2. Plaque phenotype of vM16 (*A*) and vMLN450 (*B*) viruses. Parental and recombinant viral plaques formed on HeLa cell monolayers were stained after 72-hr incubation at 37°C. Each well has a 3.5-cm diameter.

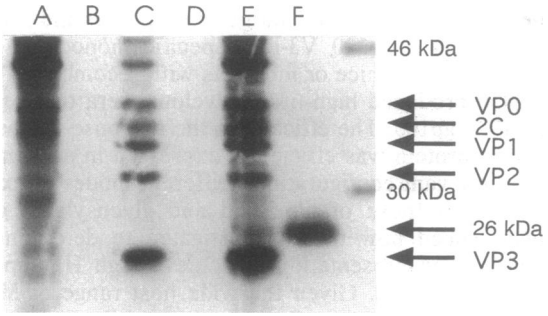

FIG. 3. Expression of Δgp120–L polypeptide in recombinant virus vMLN450-infected cells. Mock (lanes A and B), vM16 (lanes C and D), and vMLN450 (lanes E and F)-infected HeLa cells were labeled with [^{35}S]methionine. Cytoplasmic extracts were prepared at 7 hr after infection and analyzed by SDS/12% PAGE as described (12). Some samples (lanes B, D, and F) were immunoprecipitated (13) with monoclonal antibody 50.1 at 2 μg/ml before loading on the gel. Migration of Mengo virus marker proteins is indicated.

Cells infected with recombinant virus vMLN450 are expected to express a Δgp120–L fusion protein of 220 amino acids (Fig. 1). Infected HeLa cells were labeled with [^{35}S]methionine, and cytoplasmic extracts were analyzed by SDS/PAGE (12). The cells infected with recombinant virus vMLN450 produced a protein of ≈26 kDa (Fig. 3, lane E), which was clearly absent from mock- or vM16-infected cells (lanes A and C, respectively). This molecular size corresponds to the expected size of the Δgp120–L fusion protein engineered into plasmid pMRA3. The HIV-1 origin of the protein was confirmed by immunoprecipitation of labeled cell extracts (13) using monoclonal antibody 50.1, which is specific for the V3 loop of gp120 of HIV-1$_{MN}$ (Fig. 3, lane F). Expression of HIV-1-specific sequences that reacted with mAb 50.1 could also be visualized in the cytoplasm of vMLN450-infected HeLa cells by immunofluorescence analysis (data not shown).

HIV-1-Specific Antibody Response in Mice and Monkeys. To determine whether the Δgp120–L fusion protein expressed by recombinant virus vMLN450 could elicit an anti-HIV-1 antibody response, BALB/c mice were immunized twice i.p. with 10^6 pfu of live parental vM16 or recombinant virus vMLN450. Sera were collected and analyzed by direct ELISA using recombinant gp160LAI as antigen. As shown in Table 1, anti-gp160 antibodies were detected in the sera of animals immunized with recombinant virus vMLN450 but not in animals immunized with PBS or with vM16. Anti-HIV-1 titers rose ≈35-fold after the animals were boosted with recombinant virus vMLN450 and remained at a high level for at least 10 weeks after the second immunization. Similar results were obtained with CBA mice (data not shown).

HIV-1 neutralizing antibody titers were determined in a syncytia formation-inhibition assay using HIV-1$_{MN}$ (10). Neutralization titers ranged from 1:100 to 1:400 on day 70 sera from recombinant virus vMLN450-immunized BALB/c mice, whereas values for control sera were below 1:100.

Table 1. ELISA reactivity of mouse sera with HIV-1 recombinant gp160LAI

	ELISA reactivity,* titer			
Immunization (*n*)	0 days	14 days	70 days	112 days
PBS (3)	<100	<100	<100	<100
vM16 (4)	<100	<100	<100	<100
vMLN450 (4)	<100	4677	169,824	95,499

Sera of BALB/c mice were prepared at the indicated days after immunization, pooled, and tested as described.
*Titers are given as reciprocal values of the dilution giving an OD_{490} of 1.

Table 2. ELISA reactivity of monkey sera with HIV-1 recombinant gp160MN–LAI

	ELISA reactivity,* titer			
Days after immunization	Monkey 339B (vM16)	Monkey 46698 (vMLN450)	Monkey 320A (vMLN450)	Monkey 543A (vMLN450)
0	<20	43	<20	45
28	23	933	141	2089

Designations in parentheses indicate types of immunizations.
*Titers are given as reciprocal values of the dilution giving an OD_{490} of 1.

Comparable experiments were conducted with cynomolgus monkeys. Animals were immunized with a single i.m. injection of 10^6 pfu of live vM16 or recombinant virus vMLN450. HIV-1-specific antibodies were measured 1 mo later by ELISA, using either purified recombinant gp160MN–LAI (Table 2) or a commercial ELISA kit (ELAVIA; data not shown). Both tests demonstrated the presence of elevated gp160-specific antibodies in the vMLN450-immunized monkeys, whereas the vM16-immunized animal showed no reactivity.

gp120-Specific Cytotoxic Immune Response. In addition to B-cell and T-helper cell epitopes, the V3-loop sequence of gp120 of HIV-1$_{MN}$ has been shown to contain a major histocompatibility complex class I-restricted cytotoxic T-lymphocyte (CTL) epitope that is recognized in mice in the context of the H-2^d haplotype (11). We therefore searched for the presence of a cytotoxic cellular immune response toward this epitope in BALB/c mice immunized with recombinant virus vMLN450. Spleen cells from immunized mice were stimulated *in vitro* with P18-MN peptide and assayed for cytotoxic activity toward syngeneic target cells (P815) that had been pulsed with peptide P18-MN, containing the V3 CTL-epitope sequence (11). As shown in Table 3, a clear HIV-1$_{MN}$-specific cytotoxic activity could be demonstrated at an effector-to-target ratio of 25:1 in the animals immunized with recombinant virus vMLN450 but was not seen in those animals immunized with the parental vM16. As will be published in detail elsewhere, this activity was major histocompatibility complex class I-restricted.

DISCUSSION

We have demonstrated here that Mengo virus can be used as a vector for the expression of an immunogenic foreign protein sequence. In this case, 147 amino acids from the HIV-1$_{MN}$ gp120 were fused in-frame into the N terminus of the L polypeptide of the vM16 strain of Mengo virus. The recombinant virus was viable, although showing somewhat smaller plaque size and reduced virus yields as compared with vM16. The recombinant vMLN450 virus could be stably passaged for at least four cycles in cell culture with complete retention of the HIV-1 sequence. Upon further cell culture passages, revertants with deletions in the HIV-1 segment tended to

Table 3. HIV-1$_{MN}$ V3-specific cytolysis

	Specific lysis, %	
Immunization	Control P815 cells	P18-MN-pulsed P815 cells
vM16	1	4
vMLN450	3	36

Spleen cells from vM16 or vMLN450-immunized BALB/c mice were pooled and subsequently restimulated *in vitro* as described. Cytolytic activity was measured against P815 cells pulsed or not pulsed with P18-MN peptide at an effector/target ratio of 25:1.

accumulate. Whether this result is due to the 6% increase in genome length or to interference of the Δgp120 sequence with an unknown function of the natural L protein is not yet known. Possibly with additional genetic manipulation, we can optimize the size and location of the inserted segment to provide more stable and robust recombinant strains. It has been reported that the size of the poliovirus genome can be increased by up to 17% in recombinant bicistronic constructions and still be encapsidated, although with reduced viability. Poliovirus genomes 31% longer than wild type are not encapsidated (14). We do not know the maximum genomic size that can be tolerated by Mengo virus. The wild-type cardiovirus genomes usually harbor long poly(C) tracts that have been artificially truncated in vM16 to provide attenuation. Thus, it would not be surprising if the genetic capacity of vM16 were as large or even larger than that of poliovirus and perhaps as large as 1500 nt.

Our nonstructural, fusion-protein approach for the construction of live recombinant vaccines differs from that previously described with polio and rhinovirus (15–18) and may be more promising. The previous design of most recombinant picornavirus vaccines has focused on the replacement of capsid-surface antigenic loops with immunogenic segments from heterologous pathogens. This approach is necessarily limited to short peptide fragments. The antibody response elicited by such chimeras is essentially monoclonal and usually weak, presumably because of improper conformation of the inserted sequence. Moreover, such recombinants typically exhibit reduced viability and/or genetic instability. Obviously a good vector should be able to express a long amino acid sequence in a context that does not perturb virion assembly or particle stability.

The virions of vMLN450 carry an HIV-1/Mengo recombinant RNA genome, but the viral capsids are identical to those of parental Mengo virus. The HIV-1 sequence is replicated and expressed only during infection, as is typical for other Mengo nonstructural proteins. The viral 3C cleavage site between the fusion protein and P1 region is recognized and processed normally. Polyprotein translation directed by the 5′ noncoding internal ribosome entry site (19–21) is also normal, and initiated at the appropriate AUG of the fusion-protein sequence. The ability of this internal ribosome entry site to direct efficient translation of a wide variety of heterologous protein sequences is already well established (14, 22). We have, for example, recently constructed another Mengo virus recombinant encoding part of the rabies virus G protein that expresses a ΔG–L fusion protein that can be immunoprecipitated by a rabies-specific antibody (R.A., unpublished work). We have also engineered a vector cassette that will allow easy insertion of almost any antigen-encoding cDNA segment into the L protein region of an attenuated Mengo virus genome.

The cytoplasmic location of the infectious cycle of picornaviruses and the fact that picornavirus genomic RNA does not undergo reverse transcription are desirable features for any viral expression vector to be used as a live recombinant vaccine. The vM16 system could potentially show broad applicability and safety in a wide variety of mammalian hosts. Engineered deletion of the 5′ noncoding poly(C) tract has stably attenuated the virus and increased the murine LD_{50} by as much as 10^6 pfu (7). No signs of other diseases such as diabetes could be observed in immunized animals. After inoculation, the attenuated virus replicates to a limited extent in mice, pigs, and nonhuman primates but is rapidly cleared and leaves behind a lifelong, protective immunity.

The gratifying and unexpected aspect reported in this current study is that the immunogenic response to attenuated Mengo virus clearly extends to heterologous antigens that are carried and expressed by the virus during its limited replication. The Δgp120–L polypeptide protein synthesized within cells retained natural antigenic properties and could be recognized by a gp120 V3-loop-specific monoclonal antibody. Infection of mice or monkeys with recombinant virus vMLN450 produced high-titer polyclonal sera that reacted with HIV-1 gp160. The efficacy of the response means that the fusion protein was efficiently expressed in an immunologically relevant configuration. Different modes of expression, such as those of nonfused and glycosylated gp120 segments, need now to be investigated to determine the optimal antigen presentation to achieve high HIV-specific neutralization titers. Given the wide host range of Mengo virus and the strong humoral response typically elicited by its nonstructural proteins, it is likely that vM16-based recombinants expressing appropriate heterologous antigens will induce high-titer protective responses against various pathogens in many different animal hosts.

The potential interest of viral vectors for the engineering of recombinant vaccines also depends on their ability to induce a cytotoxic immune response. Although it is difficult to induce cytotoxic responses with a nonreplicating or subunit vaccine, live vectors allow correct presentation of antigens in association with major histocompatibility complex class I antigens and the efficient induction of CTL. A distinct BALB/c mice CTL epitope has been found in the V3 loop of most HIV-1 isolates (11). Our data demonstrate that recombinant virus vMLN450-immunized BALB/c mice developed an HIV-1_{MN}-specific CTL response directed against this epitope.

Our results clearly suggest that attenuated Mengo viruses have strong potential as vectors for the expression of heterologous antigens in cultured cells and as live recombinant vaccines in a wide range of animal species. In view of the ability of Mengo virus to infect human cells *in vitro* and its broad host range in animals, including nonhuman primates, it is likely that Mengo virus can also replicate in humans. Productive infection of humans has been reported for several strains of the Cardiovirus family (23, 24).

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