Proc. KSAM Fall Meeting
October 28, 2000, Chonju, Korea

Oral AIDS Vaccine Development with RPS-Vax System as a Live Vector

Yong-Soo Bae

Hannam University Dept. of Microbiology.

Poliovirus Sabin 1 strain has its own special features that make it a particularly attractive live recombinant mucosal vaccine vehicle. Sabin 1 cDNA was manipulated to have multiple cloning site and viral specific 3C-protease cutting site at the N-terminal end of the polyprotein, named RPS-vax system. HIV-1 gag, env, and their small epitope-concatamers were successfully cloned into the multiple cloning site of the vector system and produced expected chimeric viruses by transfection of their RNA transcripts into HeLa cells. These chimeric viruses have shown to express introduced vaccine genes efficiently during their replication in the infected HeLa cells. Expressed proteins were confirmed to retain the wild type structures at least in parts. Replication capacity of the chimeric viruses was slightly lower than that of wild type Sabin 1 likely to be due to delay in processing steps during their replication. Differing from the virulent Mahoney vectors, the Sabin 1- derivd RPS-Vax chimeric viruses maintained the foreign gene stably during the serial passages. These chimeric viruses have also shown to be able to induce specific humoral immunity to the introduced vaccine proteins when inoculated into the poliovirus receptor-expressing transgenic mice. Antiserum obtained from the immunized transgenic mice has a neutralizing capacity to HIV-1 *in vitro*. These results strongly suggest that the chimeric viruses expressing HIV-1 vaccine epitopes can be used as a good live mucosal vaccine candidate against AIDS.

Introduction

Several laboratories have investigated the use of poliovirus as a vector for presentation of foreign antigens. Almond and his colleagues (4, 5, 7, 9, 20) have constructed chimeric polioviruses, expressing foreign antigen on the surface capsid, by replacing surface antigenic site of VP1 with several small antigenic epitopes on the basis of the

three-dimentional crystallographic structure of poliovirus type 1 and its attenuated Sabin 1 strain. In this system, only short linear epitopes (shorter than 25 aa) can be expressed as part of the viral capsid. Wimmer's group uses dicistronic poliovirus by duplicating the 5'-noncoding internal ribosomal entry site (IRES) to express foreign antigen together with essential viral proteins (2, 11). But the instability of the inserted sequence remains to be solved. Morrow and his colleagues (1, 16, 17, 20) have suggested poliovirus minireplicon, in which poliovirus structural genes are replaced by foreign sequences, to develop poliovirus-associated mucosal vaccines. In case of minireplicon, the defective recombinant viral genome must be cotransfected with another capsid protein-expressing vector for packaging of chimeric viral genome (15).

Recently, a new strategy was suggested for expression of foreign antigens in the replication-competent recombinant polioviruses by Andino *et al* (3) and Mattion *et al.* (12). They have introduced a new protease-recognition site on the N-terminal end of the polyprotein of wild type poliovirus. According to this system, foreign gene, cloned in-frame with the poliovirus open reading frame, is followed by an artificial 3C protease site, to allow proteolytic cleavage of the foreign protein from the poliovirus polyprotein. The exogenous nucleic acid is incorporated directly into the poliovirus genome. The exogenous sequences are expressed during virus replication as part of the virus polyprotein and subsequently processed by virus-encoded proteases to produce free antigen and mature viral protein. The foreign antigen is not packaged in the virion but released into the cytoplasm. By this strategy, several HIV-1 subgenomes (3), and hepatitis surface antigen (22) were reported to be successfully expressed in poliovirus Mahoney strain, and rotavirus VP7 (12) and Pre-S2 region of hepatitis B surface antigen (6) were expressed in poliovirus Sabin 3 attenuated strain.

Our work was initiated to develop a safe poliovirus vaccine vehicle with Sabin 1. Even in a small number of cases, vaccination with oral polio vaccine (OPV) is associated with vaccine-associated paralytic poliomyelitis (VAPP), and the VAPP is most frequently associated with Sabin 2 and Sabin 3, but rarely with Sabin 1 (8, 14). Taking advantage of the strategy proposed by Andino *et al.* (3) and Mattion *et al.* (12), we have introduced a multiple cloning site and artificial 3C-recognition site at the N-terminal end and VP3/VP1 junction of the Sabin 1 open reading frame, resulting in production of recombinant progeny

viruses by transfection of HeLa cells. The recombinant viruses has a similar replication capacity and physicochemical stability to those of wild type Sabin 1. To test the usability of the recombinant Sabin 1 vector, HIV-1 p24 was cloned and expressed in this system.

Experimental Results

Construction of recombinant Sabin 1 plasmids. As shown in previous report (10), the RNA transcribed *in vitro* from poliovirus cDNA was much more infectious than cDNA itself. To increase the transfection capacity of the recombinant poliovirus cDNA, Poliovirus Sabin 1 cDNA, taken from the original pVS(1)IC-0(T) cDNA, was subcloned into a pTZ-18/R vector right after the T7 promoter site. Exogeneous sequences, containing multiple cloning site and 3C-protease recognition site, were newly inserted in frame into the region of the N-terminal or VP3/VP1 junction of the Sabin 1 cDNA, and the recombinant vectors were named pTZ-PVS-3m (recently renamed pRPS-Vax system) and pTZ-PVS-4m. The two recombinant plasmids were used to facilitate cloning of foreign genes into poliovirus genome, and processing foreign proteins from the polyprotein by viral-specific 3C-protease during the replication. The DNA fragment encoding N-terminal 169 amino acids of HIV-1 p24 was PCR amplified and cloned into the multiple cloning sites of these two vectors as illustrated in Fig. 1. Positive clones were selected by PCR for p24 fragment cloned in both multiple cloning site of Sabin 1 vectors, and then named pTZ-PVS-3m/p24 and pTZ-PVS-4m/p24, respectively. These recombinant vectors were sequenced to confirm whether the p24 fragment was cloned in frame in the vectors.

Recombinant RNA synthesized from pTZ-PVS-3m/p24 is infectious.

Full-length RNA of the p24-integrated recombinant Sabin 1 were synthesized from the p24-integrated Sabin 1 cDNA with T7 RNA polymerase after linearization with *Sal*I. These RNA transcripts were introduced into HeLa cells. Following transfection of HeLa cells with RNA transcripts, both vectors (pTZ-PVS-3m and -4m) produced recombinant viruses. Virion forming capacity of pTZ-PVS-3m RNA transcript was similar to that of wild type Sabin 1, but that of -4m was about 1 log lower than that of wild type Sabin 1 (Table 1). Whereas,

HIV-1 p24-incorporated 3m (pTZ-PVS-3m/p24) transcript produced progeny virus (PVS-3m/p24) when transfected, and the replication capacity of the recombinant chimeric virus was about 1 log lower than that of PVS-wild type Sabin 1 (Fig. 2), in the one-step growth curve, suggesting that the recombinant progeny viruses produced by transfection experiments have a little bit attenuated cytopathic effects as compared with that of wild type Sabin 1. On the other hand, pTZ-PVS-4m/p24 transcript did not produced any detectable infectious progeny virus in the same experiments.

PVS-3m/p24 recombinant chimeric virus expresses functional p24.

PVS-3m/p24 recombinant chimeric virus was tested for their capacity to express the processed p24 protein during the viral replication in HeLa cells. Not only infected HeLa cells but also supernatants harvested 18 hrs p.i. contained substantial amounts of recombinant p24 which were markedly detected by anti-p24 antisera in western blot experiments (Fig. 3). In order to see whether the expressed p24 is functionally intact, infected cells were labeled with ^{35}S-labeled amino acids, and the lysates were precipitated with AIDS patients' sera. As shown in Fig. 4, clear signal of p24 band appeared at the autoradiogram of the sample separated on SDS-PAGE. Trace amounts of unprocessed precursor bands were also detected (Fig. 5). These results suggest that the recombinant p24 expressed from the chimeric poliovirus is immunologically similar to wild type p24.

Replication capacity of PVS-3m/p24 recombinant chimeric virus. PVS-3m/p24 recombinant chimeric virus shows 1 log lower than that of wild type Sabin 1 (PVS) in their replication capacity, but was similar to that of PVS-3m recombinant virus (Fig. 5). The rate of the viral RNA synthesis was measured for Sabin 1 , PVS-3m and PVS-3m/p24 by counting the amounts of [^{3}H]-uridine incorporation into RNA. As shown in Fig. 6, the kinetics of RNA synthesis were similar for all three viruses. However, the polyprotein processing of the recombinant virus was shown relatively delayed in radioimmuniprecipitation (RIP) asssay (Fig. 4), suggesting that the attenuated one-step growth curve of PVS-3m/p24, despite the similar kinetics in RNA synthesis, is likely at least partially to be due to the slow processing and assembly of the recombinant viruses. Whereas, those one-step growth curves were denoted by

measuring the infectious virus particles. We also titrated the total progeny viruses by measuring the [^{3}H]-uridine incorporated viral RNA in the culture supernatants at each time points to see whether the lower titer of the recombinant viruses is to be associated with different ratio of defective interfering (DI) particles. As expected, one-step growth curves are similar for all of these three viruses when denoted by amounts of total viruses at each time point (data not shown). It reveals that the discrepancy of the replication capacity between the recombinant virus and wild type Sabin is likely to be due to the different amounts of defective virus particles.

Stable expression of cloned p24 during the replication of PVS-3m/p24 chimeric virus. PVS-3m/p24 chimeric virus was tested for its sequence integrity at the HIV-1 p24 cloning site by PCR and sequencing. As reported previously with Sabin 3 vector (12) we could detect shortened DNA fragments together with intact fragments in RT-PCR as the number of passages increased (Fig. 7). But, we could not find any sequence changes at the intact PCR fragments (642bp) of cloned p24 at chimeric progeny viruses during the passages. On the other hand, shortened PCR fragments (270bp) shown in Fig. 8 were found to have N-terminal 57 aa-covering region of p24 and 54-56 nucleotides of in frame or frame-shifted VP2 region by sequence analysis, and the joining shifted the reading frame of the following polyprotein of the poliovirus, causes prematured chain termination. Particularly noteworthy is that the cloned p24 (18.4 kDa) was very stably expressed from the chimeric viruses even after 12 passages in HeLa cells (Fig. 8). But, small p24 peptide (about 57 aa) shortened by internal deletion could not be detected in any experiments, such as western blot and RIP assay with anti-p24 polyclonal antibodies. We could only detect the cloned size of p24 as shown in Fig. 3 and 4. All of these data strongly support the assumption that the shortened PCR bands might be originated from the defective virus particles during the replication.

Discussion

Several investigators have tried to develop poliovirus as a mucosal vaccine vector by means of epitope substitution (4, 5, 7, 9, 20), dicistronic IRES (2, 11), poliovirus minireplicon

(1, 34, 35, 37), or autoprocessing replication-competent recombinant poliovirus (3, 12, 21). We took advantages of the strategy of an autoprocessing recombinant poliovirus, to produce Savin 1 poliovrius as a mucoal vaccine vector. Multiple cloning site and viral specific 3C-protease cutting site were newly introduced into the Sabin 1 cDNA and then followed by production of recombinant Sabin 1 poliovirus which has slightly reduced replication capacity as compared with that of wild type Sabin 1. We have tried to introduce MCS and 3C-protese recognition site into several positions of the N-terminal end. Recombinant virus was produced only when the gene was manipulated between 1st and 2nd amino acids (M/A), and VP3/VP1 junction site as shown in Fig. 1.

N-terminal 169 aa of HIV-1 p24 was cloned into the multiple cloning site of pTZ-PVS-3m and -4m recombinant vectors, and their RNA transcripts (PVS-3m/p24 and PVS-4m-p24) were introduced into HeLa cells to generate chimeric recombinant poliovirus. The RNA transcript synthesized from pTZ-PVS-3m/p24 produced chimeric virus when transfected, but that synthesized from pTZ-PVS-4m-p24 did not (Table 1). Cloning of the p24 into PVS-4m seemed to make it worse for its transfection capacity by inefficient processing at VP3/VP1 junction site. Whereas, the 3C protease accurately recognizes and cleaves the inserted synthetic proteolytic site in PVS-3m/p24, freeing the exogenous HIV-1 p24 protein from the rest of the poliovirus polyprotein. The recombinant p24 was detected at the lower band (18.4 kDa) than that of wild type p24 in western blot hybridization (Fig. 3), which means that the recombinant p24 was produced by the infection of chimeric virus, not by the contamination of the wild type p24. The recombinant p24 processed from the long polyprotein turned out to be similar for its antigenicity and structural integrity to those of wild type p24 in the western blot experiment and radioimmunoprecipitation assay with antisera (Fig.3 and Fig. 4). It means that the manners of expression and processing are expected to be applicable to any vaccine genes when introduced into the same MCS and expressed in the recombinant Sabin 1 live vaccine vehicle.

Mahoney vector (3) was reported recently for its instability of the inserted foreign gene during the passages. The rapid deletion of the inserted sequence during the passages was explained to be due to the high rate of homologous recombination in picornavirus (3, 13, 21).

Differing from the Mahoney vector, our Sabin 1 vector has shown to maintain the inserted p24 sequence even after 12 passages. In our cases, small PCR fragments were also detected at each passage together with full length of cloned p24 as shown in Fig. 7, when the progeny viruses were tested by RT-PCR with poliovirus primers (680-697/sense and 814-797/antisense). Sequencing experiments revealed that some of the small PCR fragments are artifacts which were produced by nonspecific annealing of the antisense-PCR primer. Whereas, this artifact PCR fragments lead us enable to explain the reason why the chimeric viruses have a reduced replication capacity as compared with that of wild type Sabin 1. That seems to be explained as follows. During the replication of the chimeric viruses, homologous recombination occurs to some extent in the sequences between the p24 and poliovirus, causing internal deletion, and the replication-defective viral genomes are packaged by the intact capsid proteins produced from the normal chimeric viral genome in the same cells. Therefore chimeric virus-infected cells produce substantial amounts of defective viruses together with replication-competent chimeric viruses, influencing on the reduction of the replication capacity of the chimeric viruses as shown in Fig. 5. Actually, total amounts of viral RNA synthesized in the infected cells and the amounts of the progeny viruses produced from the chimeric virus-infected cells were shown to be similar to those of wild type Sabin 1-infected cells (Fig. 6). Initiation of the exponential phase of viral RNA synthesis at about 6 hr p.i. in HeLa cells is consistant with other studies done with Vero cells for attenuated Sabin 3 viruses (12).

Recombinant poliovirus constructed from Mahoney vector rapidly deleted parts of the inserted foreign sequences during the passages, resulting in production of unwanted but replication-competent recombinant polioviruses (13, 21), and this unwanted recombinant viruses were relatively stable and exhibited improved replication capacity (13). On the contrary, our Sabin vector-derived chimeric virus produced substantial amounts of replication-competent progeny virus which maintains sequence integrity. That is thought the reason why the chimeric virus was able to maintain the expression of p24 during the passages as shown in Fig. 8.

All of our experimental results strongly suggest that the recombinant poliovirus Sabin

1 vector, developed in our laboratory, can be used as a live vaccine vector. We have constructed several chimeric polioviruses which are expressing HIV-1 env, V3-multimer, PND-concatamer, SHIV CTL epitope, HBVc-HIV-1 PND-concatamer, etc. These chimeric virues are now under immunological analysis and investigation for their immunogenicity in experimental animals.

References

1. Ansaradi, D.C., Moldoveanu, J., Porter, D.C., Walker, D.E., Conry, R.M., LoBuglio, A.F., McPherson, S., & Morrow, C.D. (1994). Characterization of poliovirus replicons encoding carcinoembryonic antigen. *Cancer Res.* **54**, 6359-6364.
2. Alexander, L., Lu, H., & Wimmer, E. (1994). Poliovirus containing picornavirus type 1 and/or type 2 internal ribosomal entry siteelements: Genomic hybrid and the expression of a foreign gene. *Proc. Natl. Acad. Sci. USA* **91**, 1406-1410.
3. Andino, R., Silvera, D., Sugget, S.D., Achacoso, P.L., Miller, C.J., Baltimore, D., & Feinberg, M.B. (1994). Engineering poliovirus as a vaccine vector for the expression of diverse antigens. *Science* **265,** 1448-1451.
4. Burke, K.L., Dunn, G., Ferguson, M., Minor, F.D., & Almond, J.W. (1988). Antigen chimeras of poliovirus as potential new vaccines. *Nature* **332**, 81-82.
5. Burke, K.L., Evans, D.J., Jenkins, O., Meredith, J., D'Souza, E.D.A., & Almond, J.W. (1989). A cassette vector for the construction of antigen chimeras of poliovirus. *J.Gen. Virol.* **70**, 2475-2479.
6. DiMichele, S., Reilly, P., Mattion, N., Fantini, S., Camposano, E., Crowley, J., Weeks-Levy, C. (1995). Characterization of recombinant poliovirus expressing the Pre-S2 region of hepatitis B surface antigen. Vaccine 95, Cold Spring Harvour laboratory Press pp 293-297.
7. Evans, D.J., McKeating, J., Meredith, J.M., Burke, K.L., Katrak, K., John, A., Ferguson, M., Minor, P.D., Weiss, R.A., & Almond, J.W. (1989). *Nature* **339**, 385-388.
8. Furione, M., Guillot, S., Otelea, D., Balanant, J., Candrea, A., & Crainic, R. (1993). Polioviruses with natural recombinant genomes isolated from vaccine-associated paralytic

poliomyelitis. *Virology* **196**, 199-208.

9. Jenkins, O., Cason, J., Burke, K.L., Lunney, D., Gillen, A., Patel, D., McKain, D.J., & Almond, J.W. (1990). An antigen chimera of poliovirus induces antibodies against human papillomavirus type 16. *J. Virol.* **64**, 1201-1206.
10. Kapalan, G., Lubinski, J., Dasgupta, A.,& Racaniello, V.R. (1985). In vitro synthesis of infectious poliovirus RNA. *Proc. Natl. Acad. Sci. USA* **82**, 8424-8428.
11. Lu, H., Alexander,L., & Wimmer,E. (1995). Construction and genetic analysis of disistronic poliovirus containing open reading frames for epitopes of human immunodeficiency virus type 1 gp120. *J. Virol.* **69**, 4797-4806.
12. Mattion N.M., Reilly, P.A., DiMichele, S.J., Crowley, J.C., & week-Levy, C. (1994). Attenuated poliovirus as a live vector : Expression of rotevirus outer capsid protein VP7 by using recombinant Sabin 3 viruses. *J. Virol.* **68,** 3925-3933.
13. Mueller, S., and Wimmer, E. (1998). Expression of foreign proteins by poliovirus polyprotein fusion: analysis of genetic stability reveals rapid deletions and formation of cardioviruslike open reading frames. *J. Virol.* **72**, 20-31.
14. Otelea, D., Guillot, S., Furione, M., Combiescu, A.A., Balanant, J., Candrea, A., & Crainic, R. (1992). Genomic modifications in naturally occuring neurovirulents of Sabin 1 polioviruses. *Dev. Biol. Stand.* **78**, 33-38.
15. Porter, D.C., Ansaradi, D.C., & Morrow, C.D. (1995). Encapsidation of poliovirus replicons encoding the complete human immunodeficiency virus type 1 gag gene by using a complementation system which provides the P1 capsid protein in trans. *J.Virol.* **69,** 1548-1555.
16. Porter, D.C., Ansaradi, D.C., Choi, W.S., & Morrow, C.D. (1993). Encapsidation of genetically engineered poliovirus minireplicons which express human immunodeficiency virus type 1 gag and pol proteins upon infection. *J. Virol.* **67**, 3712-3719.
17. Porter, D.C., Melsen, L.R., Compans, R.W., & Morrow, C.D. (1996). Release of virus-like particles from cells infected with poliovirus replicons which express human immunodeficiency virus type 1 gag. *J. Virol.* **70**, 2643-2649.
18. Porter, D.C., Wang, J., Moldoveanu, Z., McPherson, S., & Morrow, C.D. (1997). Immunization of mice with poliovirus replicons expressing the C-fragment of tetanus toxin

protects against lethal challenge with tetanus toxin. *Vaccine* **15**, 257-264.

19. Racaniello,V.R., & Baltimore, D. (1981). Cloned poliovirus complementary DNA is infectious in mammalian cells. *Science* **214**, 916-918.
20. Rose, C., Andrews, W., Ferguson, M., McKeating, J., Almond, J.W., Evans, D. (1994). The construction and characterization of poliovirus antigen chimeras presenting defined regions of the human T lymphocyte marker CD4. *J. Gen. Virol.* **75**, 969-977.
21. Tang, S., Van Rij, R., Silvera, D., and Andino, R. (1997). Toward a poliovirus-based simian immunodeficiency virus vaccine : Correlation between genetic stability and immunogenicity. *J. Virol.* **71**, 7841-7850.
22. Yoshida, Y., and Hamada, H. (1997). Adenovirus-mediated inducible gene expression through tetracyclin-controllable transactivator with nuclear localization siganl. *Biochem. Biophys. Res. Com.* **230**, 426-430.