Engineering Poliovirus as a Vaccine Vector for the Expression of Diverse Antigens

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As a step toward developing poliovirus as a vaccine vector, poliovirus recombinants were constructed by fusing exogenous peptides (up to 400 amino acids) and an artificial cleavage site for viral protease $3C^{pro}$ to the amino terminus of the viral polyprotein. Viral replication proceeded normally. An extended polyprotein was produced in infected cells and proteolytically processed into the complete array of viral proteins plus the foreign peptide, which was excluded from mature virions. The recombinants retained exogenous sequences through successive rounds of replication in culture and in vivo. Infection of animals with recombinants elicited a humoral immune response to the foreign peptides.

Important advantages of the oral poliovirus vaccine include its documented efficacy, safe use in children soon after birth, ease of administration, economy of delivery, and ability to induce mucosal immunity (*1*). The potential adaptation of poliovirus as a vaccine vector to express antigens from other pathogens has been limited by the small size of inserted sequences that can be tolerated and by the genetic instability of the chimeric viruses (*2*). The 7.5-kb genomic RNA of poliovirus directs the synthesis of a large polyprotein precursor that is ultimately cleaved by viral proteases. The viral protease $3C^{pro}$ recognizes and cleaves a characteristic amino acid sequence (AXXQG) within exposed and flexible structural domains (*3*). Using infectious poliovirus complementary DNA (cDNA) clones, we fused foreign proteins to the NH_2-terminus of the polyprotein precursor and placed a recognition site for $3C^{pro}$ at the junction (Fig. 1A). We reasoned that after synthesis of the fusion protein, the viral $3C^{pro}$ protease would cleave at the artificial cleavage site, thus releasing the foreign protein from poliovirus proteins and allowing normal viral replication.

Poliovirus chimeras were constructed by individually cloning sequences encoding five antigenic proteins into the Mahoney type I wild-type poliovirus or its Sabin-attenuated derivative (*4*). The antigens chosen, all believed to induce protective immune responses in naturally infected or experimentally immunized hosts, were the 53 COOH-terminal amino acids of *Vibrio cholerae* tcpA (the major subunit of the bacterial pilus), cholera toxin subunit B (CTB), and a 100–amino acid segment of the influenza A virus hemagglutinin (HA). Full coding sequences for the human immunodeficiency virus–type 1 (HIV-1) Nef protein (207 amino acids) and the HIV-1 p17 and p24 Gag fusion protein (363 amino acids) were also cloned into poliovirus vectors because they are important and relatively conserved targets for the cellular antiviral immune response (*5*).

All poliovirus-cDNA constructs yielded replication-competent viruses after RNA transfection in HeLa cells (*4*). Recombinant mo-hgag [carrying ~1090 nucleotides (nts) of *gag*] replicated at rates similar to the wild-type parental strain and achieved nearly equivalent titers (Fig. 1B). Recombinant mo-hnef (carrying 620 nts of *nef*), mo-pi (carrying 150 nts from *tcpA*), and mo-sb (carrying 300 nts of CTB) replicated less well (Fig. 1B). Insertion of exogenous sequences at other sites in the genome, such as at Vp1-2A, 2A-2B, and 2C-3A junctions, also yielded viable chimeric viruses;

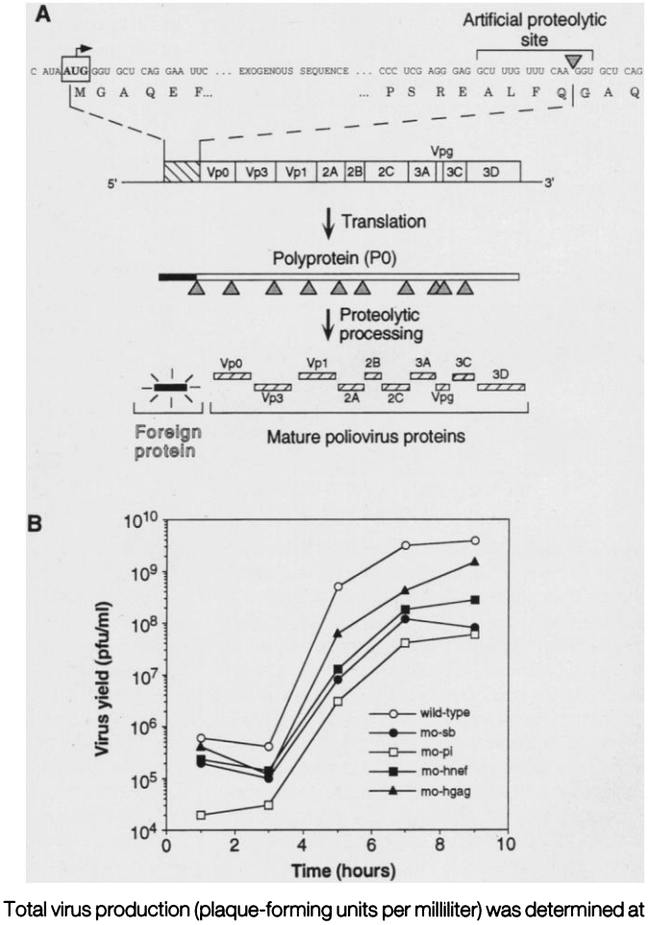

Fig. 1. (**A**) Schematic of poliovirus vectors and strategy for expression of foreign proteins. The bar represents the recombinant poliovirus genomic RNA. Viral genes are indicated within boxes; the exogenous sequences at the 5′ end of the open reading frame are indicated by a striped box. Sequences surrounding the site of insertion of the exogenous sequences are also indicated: the start codon, additional amino acids flanking the exogenous sequence that provide a restriction enzyme polylinker, the $3C^{pro}$ artificial cleavage site, and the NH_2-terminus of the viral polyprotein. Translation of the viral RNA produces an extended polyprotein, but proteolytic processing at the natural and artificial cleavage sites (indicated by triangles) results in the release of the foreign peptide (dark bar) and the generation of mature and functional viral proteins (striped bars) (*4*). (**B**) One-step growth curve of recombinant polioviruses. HeLa cell monolayers were infected with a multiplicity of infection (MOI) = 10 with wild-type poliovirus recombinants. Total virus production (plaque-forming units per milliliter) was determined at each time point by plaque assay (*24*).

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however, insertion at the Vpg-3C junction abolished viral replication (6). The recombinant viruses were genetically stable, as assessed by in situ hybridization and reverse transcription–polymerase chain reaction (RT-PCR) analyses (7). These results indicate that poliovirus can stably carry insertions representing 15% of the viral genome size.

To determine whether the recombinant viruses correctly expressed and processed viral and foreign polypeptides, we infected HeLa cells with wild-type or chimeric poliovirus and, 5 hours after infection, evaluated cell lysates by immunoblot analysis (8). Recombinant viruses were found to produce and process viral capsid proteins in a similar manner to the wild-type strain (Fig. 2, lanes 1 to 7). However, four of the five recombinants produced additional proteins: a slightly larger than normal P1 capsid precursor (lanes 3, 4, 5, and 7) and intermediate-sized polypeptides that probably represent incompletely processed recombinant polyproteins (lanes 3 to 7).

Cells infected with recombinants mo-sb, mo-ha (carrying 300 nts of the HA gene), and mo-hgag showed reduced expression of Vp2, although this protein could be detected when infected cells were harvested 9 hours after infection.

All recombinants also expressed the foreign polypeptides in the infected cells (Fig. 2, lanes 9, 11, 13, 15, and 17). Cells infected with recombinants mo-pi and mo-sb showed three distinct foreign antigens (lanes 9 and 11), whose molecular sizes suggested that they were fusions between the foreign protein and Vp0, Vp0-Vp3, or the entire P1 capsid precursor. Cells infected with recombinant mo-ha showed only one band corresponding to a fusion between the HA fragment and Vp0 (lane 13). The free polypeptides produced by the mo-pi, mo-sb, and mo-ha recombinants were too small to be visualized under our electrophoretic conditions. However, the free CTB encoded by mo-sb was readily detected by [^{35}S]methionine metabolic labeling of infected cells and subsequent immunoprecipitation with antisera to subunit B (6).

Cleavage of the foreign proteins derived from eukaryotic sources was very efficient. For example, extracts from cells infected with recombinants mo-hgag and mo-hnef displayed only single immunoreactive peptides corresponding to free Gag and Nef (Fig. 2). Although these viruses carry inserts that are two to three times as large as those in the other recombinants, they replicate with nearly wild-type kinetics, suggesting that the efficiency of cleavage may be an important determinant of replicative ability. Importantly, the amount of free HIV-1 Gag [quantified by an antigen capture enzyme-linked immunosorbent assay (ELISA) (Coulter)] reached 100 ng per 10^5 cells by 7 hours after infection; this represents ~10 times more Gag than is produced in the same time period by the H9 T cell line chronically infected with HIV-1.

The kinetics of polyprotein synthesis and processing were analyzed by pulse-chase experiments with recombinants mo-pi and mo-sb. The recombinants produced the vi-

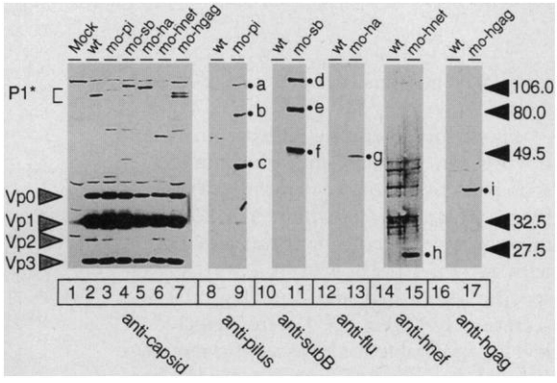

Fig. 2. Expression and processing of the exogenous proteins in cells infected with recombinant polioviruses. Cytoplasmic lysates prepared 5 hours after infection from HeLa cells infected with wild-type (wt) poliovirus or various recombinant viruses were analyzed by immunoblotting (8) with antibodies directed against poliovirus virions (lanes 1 to 7), tcpA (lanes 8 and 9), CTB (lanes 10 and 11), HA (lanes 12 and 13), HIV-1 Nef (lanes 14 and 15), and HIV-1 Gag (lanes 16 and 17). Poliovirus capsid proteins are indicated by shaded triangles. A larger version of the capsid protein precursor (P1*) is observed in recombinant-infected cells. On the basis of molcular size, the bands detected by antibodies against the inserted sequences are (a) pilus-P1 fusion; (b) pilus-Vp0-Vp3 fusion; (c) pilus-Vp0 fusion; CTB fused to (d) P1, (e) Vp0-Vp3, and (f) Vp0; (g) HA fused to Vp0; and the free HIV-1 Nef (h) and Gag (i) proteins (8). Molecular size standards are indicated to the right in kilodaltons.

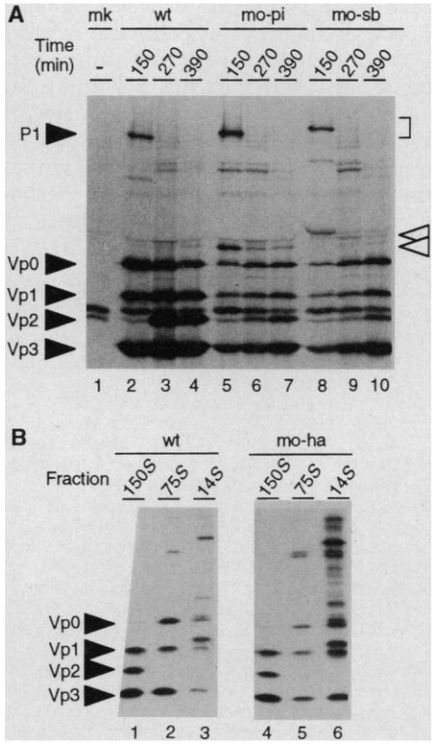

Fig. 3. Kinetics of proteolytic processing and virion assembly in infected cells. (**A**) HeLa cells were infected with wild-type (wt) or recombinant virus. After 2 hours, cells were pulse-labeled for 30 min with [^{35}S]methionine and then chased with unlabeled L-methionine. Lysates were prepared at the indicated times and subjected to immunoprecipitation with antisera to poliovirus virions. Lane 1, mock-infected (mk); lanes 2 to 4, wt-infected; lanes 5 to 7, tcpA recombinant–infected; lanes 8 to 10, CTB recombinant–infected (9). Additional processing intermediates are indicated by shaded arrows on the right. The poliovirus capsid precursor (P1) and mature proteins Vp0, Vp1, Vp2, and Vp3 are indicated by solid arrows [note that recombinant viruses produced a slightly larger P1 precursor (bracket)]. (**B**) Analysis of poliovirus virion formation. Metabolically labeled extracts from HeLa cells infected with wt or recombinant mo-ha were subjected to sucrose gradient sedimentation (9). Samples from gradient fractions corresponding to 150*S*, 75*S*, and 14*S* were analyzed by 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Mature virions (150*S*) were assembled by the recombinant mo-ha and contained the three major virion constituents, VP1, VP2, and VP3 (lanes 1 and 4). The 75*S* procapsids were composed of VP0, VP1, and VP3 (lanes 2 and 5), as were the 14*S* pentamers (this fraction also included other proteins, lanes 3 and 6).

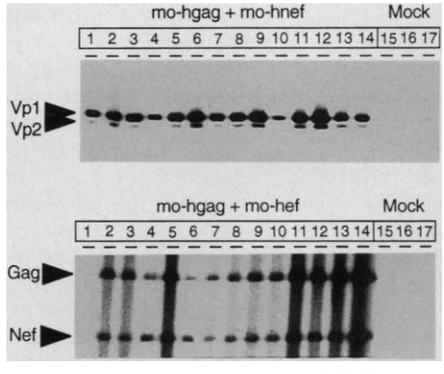

Fig. 4. Immunological response of PVR transgenic mice infected with a mixture of mo-hgag and mo-hnef (12). Sera from three mock-infected mice (lanes 15 to 17) and from 14 mice infected with the recombinants (lanes 1 to 14) were analyzed by immunoblotting (12). Bands corresponding to poliovirus capsid proteins Vp1 and Vp2 and to HIV-1 Gag and Nef are indicated.

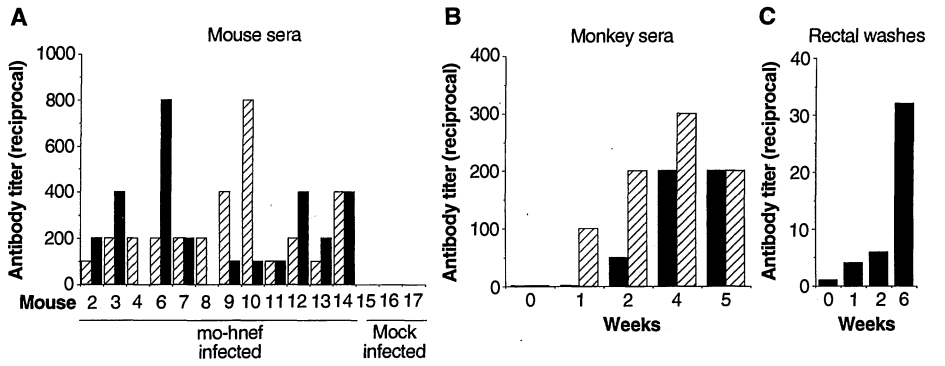

Fig. 5. Antibody titers in sera of infected mice (**A**) and in sera (**B**) and rectal washings (**C**) of an infected cynomolgus monkey. Antibody titers of anti-Nef [IgG (striped bars) or IgA (black bars)] in mouse sera were determined by ELISA (*16*) after two intraperitoneal inoculations with mo-hnef (*12*). (B and C) Anti-Nef IgG (striped bars) and IgA (black bars) titers were measured weekly in the serum and rectal washings of a monkey immunized by a single rectal inoculation with poliovirus recombinant mo-hnef (*13*).

ral capsid proteins with similar kinetics as wild-type virus (Fig. 3A). As expected, a larger P1 precursor was synthesized by mo-pi and mo-sb recombinants (Fig. 3A, compare lane 2 with lanes 5 and 8), but the precursor was cleaved to the mature proteins with kinetics similar to wild type, although some partially processed intermediates were detected (Fig. 3A, shaded arrows).

Poliovirus particle assembly proceeds through several subviral intermediates that can be resolved by sedimentation through sucrose gradients: 14S pentamers, 75S procapsids, and 150S virions. Because the foreign proteins were initially fused to the viral structural protein precursors, we examined whether the recombinant viruses assembled normally. Sucrose gradient analysis of infected cell lysates (*9*) demonstrated that the sedimentation coefficients and protein composition of mo-ha virions and viral subparticles were indistinguishable from those of wild type (Fig. 3B), suggesting that poliovirus assembly is normal and that the HA fragment is not included in the virus particles. The buoyant density in cesium chloride of mo-hgag and mo-hnef was identical to that of wild-type poliovirus, and no immunoreactive Gag or Nef was detected in purified virions (*10*).

The immunogenicity of the poliovirus recombinants was evaluated in transgenic mice that express the human poliovirus receptor (PVR) and therefore are susceptible to poliovirus infection (*11*). Mice were inoculated intraperitoneally on two occasions with a mixture of recombinants mo-hgag and mo-hnef or with saline. Serum antibodies recognizing poliovirus, and HIV-1 proteins were detected by immunoblot analysis 4 weeks after the second inoculation in all infected mice (Fig. 4) (*12*). Furthermore, the mouse sera were found to contain immunoglobulin G (IgG) and IgA to Nef (anti-Nef) at titers of 1:100 to 1:800 (Fig. 5A).

Finally, to determine if recombinant poliovirus can stimulate mucosal immune responses in primates, we performed a preliminary experiment in which a cynomolgus monkey was infected by rectal inoculation with mo-hnef (*13*). Within 2 weeks, Nef-specific serum IgG and IgA (Fig. 5B) and secretory IgA (Fig. 5C) were detected at levels comparable to those seen in monkeys (*6*) and humans (*1*) immunized with live-attenuated poliovirus vaccines.

Poliovirus recombinants may ultimately allow simultaneous vaccination against multiple pathogens through preparation of a "cocktail" of poliovirus recombinants carrying antigenic determinants derived from these pathogens. Mucosal immune responses induced by poliovirus vectors may limit pathogen replication at the portal of entry, although this hypothesis remains to be tested. In principle, similar vaccine vectors could also be derived from attenuated strains of other viruses that use proteolytic processing mechanisms, such as other picornaviruses or flaviviruses.

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4. We used the Mahoney type 1 wild-type strain (*14*) and the Sabin type 1 vaccine strain of poliovirus (*3*). The poliovirus genomes were further modified to include an in-frame synthetic polylinker containing Eco RI and Xho I sites to permit insertion of foreign sequences. At its 3′ border, the inserted segment includes sequences encoding the recognition and cleavage site for the poliovirus 3C protease (AXXQG) (*3*, *15*), introduced by PCR (*16*). The first of two PCR reactions were performed with oligonucleotide primers 1 and 2, and 3 and 4 (*17*) to amplify portions of the poliovirus genome corresponding to nucleotides 1 to 740 and 740 to 1582. The second PCR amplification was performed with oligonucleotides 1 and 4. The 1582–base pair (bp) DNA product was digested with Sal I and Aat II and ligated to a full-length poliovirus DNA Mahoney strain clone pSR-XpA (*18*); the modified plasmids were denoted pMoV-1.1. A later vector version (pMoV-1.3) carries a more extensive polylinker (including Not I and BssH II) and a polyglycine tract to provide greater flexibility to the polyprotein at the artificial cleavage site. Exogenous DNA sequences were amplified by PCR with primers (*17*) that include convenient restriction sites to facilitate insertion in the poliovirus vector plasmid. Coding sequences for CTB were amplified with primers 5 and 6 with plasmid pJM17 as a template (*19*); *V. cholerae* pilus tcpA (amino acids 156 to 199) was obtained from plasmid pRT198 (*20*) with primers 7 and 8; HA (amino acids 109 to 208) was amplified with primers 9 and 10 and the plasmid pMT2-HA (*21*); the complete sequence corresponding to p17 and p24 of the HIV-1 *gag* gene and the entire *nef* coding sequence were amplified from a derivative of pHXB2 with a repaired *nef* gene (*22*) with primers 11 and 12, and 13 and 14, respectively. PCR fragments were digested with appropriate restriction enzymes and ligated to digested pMoV-1.1 or pMoV-1.3. Replication-competent poliovirus was recovered by transfection of in vitro–synthesized recombinant poliovirus RNA into HeLa S3 cells (*23*).
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7. Recombinant mo-hnef was sequentially passed six times in HeLa cells with a MOI = 1. The final viral stock was analyzed by plaque hybridization. HeLa cells were infected with ~100 plaque-forming units (pfu) and overlaid with agar. An imprint of the plaques was obtained by layering a nitrocellulose filter onto the surface of the agar. Filters were hybridized with specific oligonucleotide probes to detect the 5′-(5′-GGT GCG AGA GCG TCA GTA TTA AGC G-3′) and 3′ ends of HIV *gag* insertion (5′-CAA AAC TCT TGC CTT ATG GCC GGG T-3′). A duplicate filter was similarly analyzed for poliovirus wild-type sequences (5′-ATG CAG CGA CGT CTG GTT CTG TCG-3′). RT-PCR analyses of purified mo-hgag and mo-hnef virion RNA were performed to determine the length of the inserted sequences after six passages (*16*).
8. HeLa cells were infected with recombinant or wild-type viruses (MOI = 10) for 5 hours at 37°C. Lysates were obtained (*24*), and 4 μg of total lysates were subjected to 10% SDS-PAGE. Antisera to poliovirus was prepared in rabbits; antisera to Nef and Gag were obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, NIH; other antisera were gifts. Immunodetection was performed with an ECL chemiluminescence detection kit (Amersham).
9. HeLa cells were mock infected with poliovirus at a MOI = 10 at 37°C and placed in 2 ml of methionine-depleted Dulbecco's modified essential medium (DMEM). Two hours later, the medium was replaced with DMEM containing 25 μCi of [^{35}S]methionine per milliliter. After 30 min, L-methionine was added to a final concentration of 30 μg/ml, and the cells were incubated at 37°C for 0, 120, or 240 min before lysis and immunoprecipitation (*24*). Extracts were prepared (*8*) from infected HeLa cells labeled for 4 hours with [^{35}S]methionine. Sucrose gradient sedimentation was performed (*25*) and 10-μl fractions were analyzed by 10% SDS-PAGE.
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HeLa cells by infection with a MOI = 1. Infected cells were incubated for 8 hours at 37°C, washed with phosphate-buffered saline (PBS), and lysed by freeze-thawing in 1.5 ml of PBS. Mice were infected by intraperitoneal injection with recombinant poliovirus (100 μl of 2 × 10^8 pfu per milliliter of stock) or mock infected with PBS. PVR-transgenic mice received two identical injections separated by a period of 30 days. Four weeks later, mice were bled and their sera tested for the presence of specific antibodies. Purified HIV-1 Gag and Nef (produced in *Escherichia coli*, 3 μg each) or poliovirus-infected HeLa cell lysate (100 μg) was subjected to 10% SDS-PAGE in a single wide lane and transferred to nitrocellulose. Sera (diluted 1:200) from immunized animals were loaded into independent lanes of a multislot apparatus (Mini-protean II, MultiScreen apparatus, Bio-Rad). Antibodies reacting with the HIV-1 and poliovirus proteins were visualized with secondary antibodies to mouse Ig (anti–mouse Ig) (Amersham). ELISA was performed as described (*16*) with anti-mouse Ig secondary antibodies (Amersham).

13. A cynomolgus monkey was infected by direct deposition of single doses of 10^7 pfu of mo-hnef in 2 ml of saline. Topical rectal administration was carried out with soft, lubricated pediatric nasogastric tubes. Blood was collected weekly from femoral vessels, and the serum was separated. Rectal washings were obtained atraumatically by flushing 2 ml of PBS into the rectum and collecting the fluid with the aid of lubricated pediatric nasogastric tubes. Examination of rectal and serum antibodies was performed by ELISA (*16*). Plates coated with antigen (recombinant Nef at 5 μg/ml) were incubated with doubling dilutions of test samples. Bound antibody was detected by incubation with antibodies to human Ig and IgA secondary antibodies conjugated to horseradish peroxidase (Amersham and Zymed Labs), followed by determination of enzymatic activity with ABTS tablets (Boehringer). The absorbance was measured at 405 nm. Results are expressed as the reciprocal of the lowest dilution that gave an absorbance of 0.1 to 0.15 U above the background. Controls included assay plates coated with bovine serum albumin and preimmune samples. The reproducibility of the ELISA after three repeated assays of the same sample of serum or rectal washings was within one dilution.
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Prophylactic Vaccines, Risk Behavior Change, and the Probability of Eradicating HIV in San Francisco

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Theory is linked with data to assess the probability of eradicating human immunodeficiency virus (HIV) in San Francisco through the use of prophylactic vaccines. The necessary vaccine efficacy levels and population coverage levels for eradication are quantified. The likely impact of risk behavior changes on vaccination campaigns is assessed. The results show it is unlikely that vaccines will be able to eradicate HIV in San Francisco unless they are combined with considerable reductions in risk behaviors. Furthermore, if risk behavior increases as the result of a vaccination campaign, then vaccination could result in a perverse outcome by increasing the severity of the epidemic.

Mass vaccination campaigns against HIV will be initiated after vaccine efficacy has been established by phase III clinical trials. However, before the vaccination campaigns begin it is important to determine the potential epidemiological impact of the vaccines. Previously we have formulated and analyzed a transmission dynamic model of HIV in order to develop a quantitative framework for assessing the utility of prophylactic vaccines for epidemic control (that is, for eradication and for noneradicating control) (*1*). Here we link this theoretical work with a specific data set to assess whether it will be possible to eradicate HIV in San Francisco through the use of prophylactic vaccines. Data from the San Francisco Young Mens Health Study (SFYMHS), which is an HIV transmission study of young gay men, were used in the analysis. Specifically for San Francisco, three questions were addressed: (i) What proportion of the young gay community would have to be vaccinated in order to eradicate HIV, (ii) how effective would the vaccines have to be to ensure epidemic eradication, and (iii) what effects could changes in sexual risk behavior have on the impact of mass vaccination programs?

Vaccine efficacy is generally calculated with clinical trial data and a standard definition of efficacy, where efficacy is defined as 1 − relative risk. Incidence rates determine the magnitude of the relative risk between the vaccinated and the placebo groups; hence, the calculated efficacy level will depend on the length of the clinical trial (that is, efficacy is time-dependent) (*2–4*). Furthermore, the standard definition of vaccine efficacy does not provide a means for including the specific mechanisms of vaccine failure. Hence, previously we formulated a new measure of efficacy (that we named vaccine impact) so that we could (i) examine the effects of specific mechanisms of vaccine failure and (ii) derive a time-independent summary measure of vaccine imperfection (that is, efficacy) that could be used to calculate the critical vaccination coverage required for epidemic eradication (*1*). This new measure of efficacy was derived while the effects of mass vaccination programs were modeled on the transmission dynamics of HIV in a gay community. The transmission model consisted of four ordinary differential equations; the model structure is described elsewhere (*1*). Our model included three mechanisms of vaccine failure: take (the fraction of vaccine recipients in whom the vaccine has any immunological effect at all), degree (the degree of reduction in susceptibility per sexual partnership for those in whom the vaccine takes), and duration (the duration of vaccine-induced immunity) (*1*). We named our efficacy measure the impact of the vaccine (ϕ) (therefore efficacy and impact are synonyms) (*1*); throughout this

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