ed 10- to 100-fold less than in untreated mice but still at least 10-fold more than in IFN-α–treated control mice (*8*). These preliminary results indicate that in vivo, IFN-γ and IFN-α/β may exert their antiviral activity through different, in part nonredundant, pathways.

Interferon-γ has been proposed to regulate antigen processing at the level of both proteolytic generation (*24*) and transport of MHC class I–binding peptides (*25*). The finding that IFN-γR–deficient mice mounted normal T or B cell responses suggests that these IFN-γ effects are not essential. Still, several questions regarding the role of IFN-γ in modulating certain immune reactions remain to be answered. Thus, the balance between T helper cell subsets (*26*) known to determine the immune response to parasites such as *Leishmania* (*27*) may be affected in IFN-γR$^{0/0}$ mice. IFN-γ has been implicated in autoimmunity (*28–30*). Investigating the consequences of a genetic IFN-γR defect in these immune reactions will require backcrossing of the mutation into appropriate genetic backgrounds. In addition to its direct analytical potential, the approach of genetically inactivating the IFN-γ receptor offers the possibility of cell- or tissue-specific reconstitution of a functional receptor to further elucidate IFN-γ–mediated functions. Moreover, combining the IFN-γR defect with other cytokine deficiencies through appropriate breeding of mice may help to reveal interactions of various cytokines in vivo.

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35. Macrophages were collected from the peritoneal cavity of mice 4 days after intraperitoneal injection of 2 ml of 4% thioglycollate broth. Adherent cells were incubated at 37°C with or without lipopolysaccharide (from *Escherichia coli* 0127:88, Sigma), recombinant murine IFN-γ (10^7 U/mg, Genzyme, Cambridge, MA), or natural murine IFN-α/β (9.8×10^6 U/mg, Lee Biomolecular, San Diego, CA). After 48 hours of incubation, the NO_2^- concentration was determined.
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Heterologous Protection Against Influenza by Injection of DNA Encoding a Viral Protein

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Cytotoxic T lymphocytes (CTLs) specific for conserved viral antigens can respond to different strains of virus, in contrast to antibodies, which are generally strain-specific. The generation of such CTLs in vivo usually requires endogenous expression of the antigen, as occurs in the case of virus infection. To generate a viral antigen for presentation to the immune system without the limitations of direct peptide delivery or viral vectors, plasmid DNA encoding influenza A nucleoprotein was injected into the quadriceps of BALB/c mice. This resulted in the generation of nucleoprotein-specific CTLs and protection from a subsequent challenge with a heterologous strain of influenza A virus, as measured by decreased viral lung titers, inhibition of mass loss, and increased survival.

A challenge to the development of vaccines against viruses such as influenza A or human immunodeficiency virus (HIV), against which neutralizing antibodies are generated, is the diversity of the viral envelope proteins among different isolates or strains. Because CTLs in both mice and humans are capable of recognizing epitopes derived from conserved internal viral proteins (*1*) and are thought to be important in the immune response against viruses (*2*), efforts have been directed toward the development of CTL vaccines capable of providing heterologous protection against different viral strains. $CD8^+$ CTLs kill virally infected cells when their T cell receptors recognize viral peptides associated with major histocompatibility complex (MHC) class I molecules (*3*). These peptides are derived from endogenously synthesized viral proteins, regardless of the protein's location or function in the virus. Thus, by recognition of epitopes from conserved viral proteins, CTLs may provide cross-strain protection. Peptides capable of associating with MHC class I molecules for CTL recognition originate from proteins that are present in or pass through the cytoplasm or endoplasmic reticulum (*4*). Therefore, in general, exogenous proteins,

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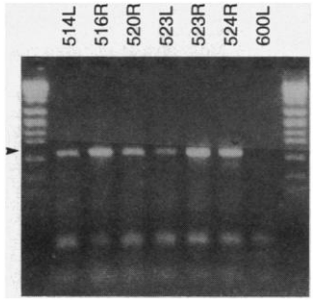


Fig. 1. Detection of NP plasmid DNA in muscle by PCR. BALB/c mice were injected three times with RSV1-NP DNA or blank vector (100 μg per leg) into both quadriceps muscles, followed by influenza infection. The muscles were removed 4 weeks after the final injection and immediately frozen in liquid N_2. They were then pulverized in lysis buffer [25 mM tris-H_3PO_4 (pH 8), 2 mM CDTA (trans-1:2-diamenocyclohexantetra–acetic acid); 2 mM dithiothreitol (DTT), 10% glycerol, and 1% Triton X-100] in a Mikro-Dismembrator (Braun Instruments), and high molecular weight DNA was extracted by phenol-chloroform and ethanol precipitation. We performed a 40-cycle PCR reaction (*32*) to detect the presence of NP plasmid DNA in muscle. A 772-bp PCR product (indicated by the arrowhead), generated from oligonucleotides spanning 18 bases of the 3′ portion of the promoter region (GTGTGCACCTCAAGCTGG) and 23 bases of the 5′ portion of the inserted NP sequence (CCCTTTGAGAATGTTGCACATTC), is seen on an ethidium bromide–stained agarose gel in selected NP DNA–injected muscle samples but not in the blank vector control (600L). Labeling above each lane indicates mouse identification number and right or left leg.

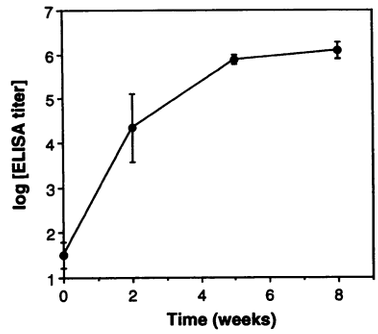


Fig. 2. Production of NP-specific antibodies in mice injected with NP DNA. Mice were injected with 100 μg of V1-NP DNA in each leg at 0, 3, and 6 weeks, and blood was drawn on 0, 2, 5, and 8 weeks. The presence of anti-NP IgG in the serum was assayed by an ELISA (*19*), with NP purified from insect cells that had been transfected with a baculovirus expression vector. The results are plotted as mean log[ELISA titer] ± SEM (n = 10) against time after the first injection of NP DNA. Mice immunized with blank vector generated no detectable NP-specific antibodies.

which enter the endosomal processing pathway (as in the case of antigens presented by MHC class II molecules), are not effective at generating $CD8^+$ CTL responses.

Most efforts to generate CTL responses have either used replicating vectors to produce the protein antigen in the cell (*5*) or have focused on the introduction of peptides into the cytosol (*6*). Both of these approaches have limitations that may reduce their usefulness as vaccines. Retroviral vectors have restrictions on the size and structure of polypeptides that can be expressed as fusion proteins and still maintain the ability of the recombinant virus to replicate (*7*), and the effectiveness of vectors such as vaccinia for subsequent immunizations may be compromised by immune responses against the vectors themselves (*8*). Also, viral vectors and modified pathogens have inherent risks that may hinder their use in humans (*9*). Furthermore, the selection of peptide epitopes to be presented is dependent on the structure of an individual's MHC antigens, and peptide vaccines may therefore have limited effectiveness due to the diversity of MHC haplotypes in outbred populations (*1–3*). Hence, immunization with nonreplicating plasmid DNA encoding viral proteins may be advantageous because no infectious agent is involved, no assembly of virus particles is required, and determinant selection is permitted. Because the sequence of nucleoprotein (NP) is conserved among various strains of influenza (*10*), protection was achieved here against subsequent challenge by a virulent strain of influenza A that was heterologous to the strain from which the gene for NP was cloned.

Intramuscular (i.m.) injection of DNA expression vectors in mice has been demonstrated to result in the uptake of DNA by the muscle cells and expression of the protein encoded by the DNA (*11*). Plasmids were shown to be maintained episomally and did not replicate. Subsequently, persistent expression was observed after i.m. injection in skeletal muscle of rats, fish, and primates and in cardiac muscle of rats (*12*). This technique could be a method of introducing viral proteins into the antigen-processing pathway that results in the generation of virus-specific CTLs. We therefore evaluated the ability of injected cDNA encoding the conserved viral protein influenza A NP to generate specific CTLs and also evaluated the protective efficacy of this approach against subsequent viral challenge.

BALB/c mice were injected in the quadriceps of both legs with plasmid cDNA encoding A/PR/8/34 NP (*13*) driven by either a Rous sarcoma virus (RSV) or cytomegalovirus (CMV) promoter (*14*). Negative control animals were uninjected or injected with the appropriate blank vector lacking the inserted NP gene. The presence or absence of NP plasmid DNA in the muscles of selected animals was analyzed by polymerase chain reaction (PCR) (Fig. 1). Plasmid DNA (either NP or luciferase DNA) was detected in 44 of 48 injected muscles tested. In mice injected with luciferase DNA, protein expression was demonstrated by luciferase activity recovered in muscle extracts (*11, 12*). NP expression in muscles after injection of NP DNA was below the limit of detection for protein immunoblot analysis (<1 ng) but was indicated by the production of NP-specific antibodies (Fig. 2). For analysis of NP-specific CTL generation, spleens were removed 1 to 4 weeks after immunization (*15*), and spleen cells were re-stimulated with recombinant human interleukin-2 (IL-2) plus autologous spleen cells that had been either infected with influenza A (A/PR/8/34) or pulsed with the H-$2K^d$–restricted NP peptide epitope (*15*) (NP residues 147 to 155) (*16*). Spleen cells re-stimulated with virally infected (*17*) cells or with epitope-pulsed syngeneic cells were capable of killing NP epitope–pulsed target cells (Fig. 3A). This indicates that i.m. injection of NP DNA generated the appropriate NP-derived peptide in association with MHC class I molecules for induction of the specific CTL response. These CTLs were capable of recognizing and lysing virally infected target cells (Fig. 3B), or target cells pulsed with the H-$2K^d$–restricted NP peptide epitope. This demonstrates their specificity as well as their ability to detect the epitope generated naturally in infected cells.

A more stringent measure of immunogenicity of the NP DNA vaccine was the evaluation of the primary CTL response. Spleen cells taken from NP DNA–injected mice were activated by exposure to concanavalin A (Con A) and IL-2 but did not undergo in vitro re-stimulation with antigen-expressing cells before their ability to kill appropriate targets was tested. Splenocytes from mice immunized with NP DNA, when activated with Con A and IL-2 in vitro without antigen-specific re-stimulation, lysed both epitope-pulsed and virally infected target cells (Fig. 3, C and D). This lytic activity of both the re-stimulated and activated spleen cells compares favorably with that of similarly treated splenocytes derived from mice that had been first infected with influenza A/HK/68, a virulent, mouse-adapted H3N2 strain that arose 34 years after A/PR/8/34 (H1N1). Thus, injection of NP DNA generated CTLs that were specific for the NP epitope and that were capable of identifying the naturally processed antigen.

Injection of mice with NP DNA resulted in the production of high-titer antibodies to

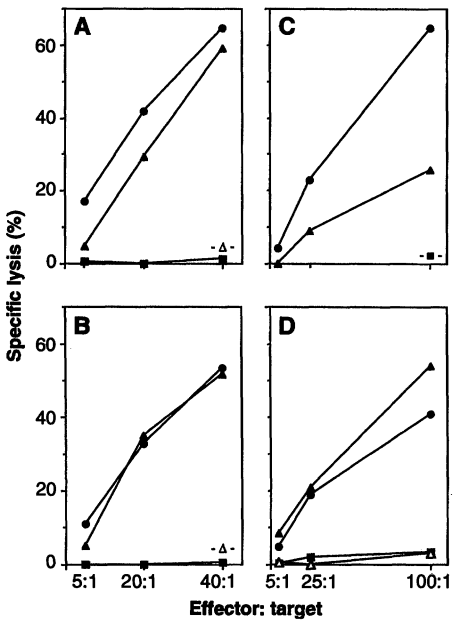
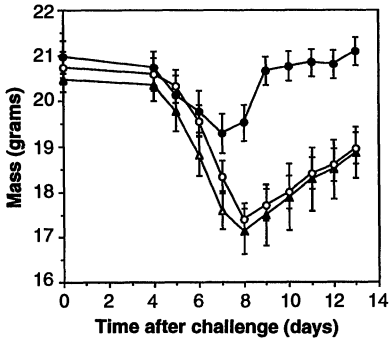


Fig. 3. Percent specific lysis determined in a 4-hour ^{51}Cr release assay (*15*) for CTLs obtained from mice immunized with DNA. Mice were immunized with 400 μg of V1-NP DNA (solid circles) or blank vector (solid squares) and killed 3 to 4 weeks later. Negative control CTLs were obtained from a naïve mouse (open triangles), and positive controls from a mouse that had recovered from infection with A/HK/68 4 weeks before (solid triangles). Graphics depict data from representative individual mice. At least eight mice were studied for each set of conditions. (**A**) Spleen cells re-stimulated with NP(147–155)–pulsed autologous spleen cells (*15*) and assayed against NP(147–155)–pulsed P815 cells. (**B**) Spleen cells re-stimulated with NP(147–155)–pulsed autologous spleen cells and assayed against P815 targets infected with influenza A/Victoria/73 (H3N2) for 6 hours before addition of CTLs. (**C**) Spleen cells re-stimulated with Con A and IL-2 without additional antigen and assayed against P815 cells pulsed with NP(147–155). (**D**) Mice were injected with 200 μg per injection of V1-NP DNA or blank vector three times at 3-week intervals. Spleens were harvested 4 weeks after the last immunization, spleen cells were cultured with IL-2 and Con A for 7 days, and CTLs were assayed against P815 target cells infected with influenza A/Victoria/73.

Fig. 4. Mass loss (in grams) and recovery in DNA-immunized mice after unanesthetized intranasal challenge with 10^4 $TCID_{50}$ of A/HK/68. Mice were immunized three times at 3-week intervals with V1-NP DNA or blank vector or were not injected and were then challenged 3 weeks after the last immunization. Masses for groups of ten mice were determined at the time of challenge and daily from day 4 for NP DNA–injected mice (solid circles), blank vector controls (open triangles), and uninjected controls (open circles). Shown are mean masses ± SEM. NP DNA–injected mice displayed significantly less mass loss on days 8 through 13 than blank vector–injected mice ($P \leq 0.005$) and uninjected mice ($P \leq 0.01$), as analyzed by the t test. No significant difference was noted between the two controls ($P = 0.8$ by the t test).



NP [anti-NP immunoglobulin G (IgG)] (Fig. 2). Generation of high-titer IgG in mice is thought to require $CD4^+$ T helper cells (*18*, *19*). This suggests that NP expressed from the plasmid in situ was processed for presentation by both MHC class I and class II molecules. To investigate a potential role of NP-specific antibodies in protective immunity to influenza, we used two approaches. First, viral lung titers were determined in a passive transfer experiment (*20*) where naïve mice were infused with anti-NP serum and then challenged with A/HK/68 (*21*). No reduction in viral lung titers was seen in mice that had received anti-NP serum, obtained from mice that were injected with NP DNA (6.3 ± 0.2; mean ± SEM; $n = 4$), as compared with control mice that had received normal serum (6.1 ± 0.3; mean ± SEM; $n = 4$). As a positive control, serum was collected from mice that had been infected with A/HK/68 and passively transferred to four naïve mice. After a challenge with A/HK/68, no viral infection was detectable in their lungs, indicating that this serum against whole virus was completely protective for challenge with the homologous virus. Second, naïve mice were immunized with purified NP (5 μg per leg; three times over a period of 6 weeks) by i.m. injection. These mice generated high-titer NP-specific antibodies but did not produce NP-specific CTLs and were not protected from a lethal dose of virus (*22*). Therefore, unlike the neutralizing effect of antibodies to whole virus, circulating anti-NP IgG did not confer protective immunity to the mice.

We evaluated the in vivo protective efficacy of NP DNA injections to determine whether a cell-mediated immune response was functionally significant. One direct measure of the effectiveness of the immune response was the ability of mice first immunized with NP DNA to clear a progressive, sublethal lung infection with a heterologous strain of influenza (A/HK/68; H3N2) (*21*). Mice immunized with NP DNA had viral lung titers after challenge that were three orders of magnitude lower on day 7 (1.0 ± 1.0; mean ± SEM; $n = 4$) than those of control mice that had not been immunized (4.1 ± 0.3; mean ± SEM; $n = 4$) or that had been immunized with blank vector (4.5 ± 0.0; mean ± SEM; $n = 4$). In fact, three of four immunized mice had undetectable amounts of virus in their lungs, whereas none of the controls had cleared virus at this point. The substantial difference in the viral lung titers seen in this experiment and six others (*22*) demonstrates that the immune response accelerated clearance of the virus. The lack of protective effect of the blank vector control confirms that DNA per se was not responsible for the immune response. Moreover, because the challenge strain of virus, A/HK/68 (H3N2), was heterologous to the strain A/PR/8/34 (H1N1) from which the NP gene was cloned, immunity was heterotypic.

To provide a measure of virus-induced morbidity, we observed the mass loss in mice that were infected sublethally with influenza A/HK/68 after immunization with NP DNA (Fig. 4). Uninjected mice or mice injected with the blank vector were used as controls. Mice immunized with NP DNA exhibited less mass loss and a more rapid return to their masses before challenge after influenza A infection, as compared with control mice.

Intranasal infection of fully anesthetized mice with influenza A causes rapid, widespread viral replication in the lung and death in 6 to 8 days if the infection is not controlled (*23*). Survival of mice challenged by this method reflects their ability to limit the severity of an acute lung infection. The capacity of mice to survive challenge with A/HK/68 (Fig. 5) and A/PR/8/34 (*24*) was studied. Mice immunized with NP DNA showed a 90% survival rate, as compared with a 0% survival rate in animals injected with blank vector and a 20% survival rate in uninjected control animals (Fig. 5). In a total of 14 such studies, mice immunized with NP DNA showed ≥50% greater survival rate than controls (*22*, *24*). Thus, the ability of the NP DNA–induced immune response to effectively accelerate recovery and decrease disease caused by a virus of a different strain arising 34 years later supports the rationale of targeting a conserved protein for the generation of a CTL response.

The i.m. injection of a DNA expression vector encoding a conserved, internal protein of influenza A resulted in the generation of significant protective immunity against subsequent viral challenge. In particular, NP-specific antibodies and primary CTLs were produced. NP DNA immunization resulted in decreased viral lung titers, inhibition of mass loss, and increased survival, as compared with controls. The protective immune response was not mediated

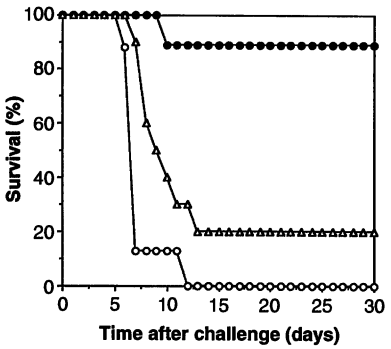


Fig. 5. Survival of DNA-immunized mice after intranasal challenge (under anesthesia) with $10^{2.5}$ $TCID_{50}$ of A/HK/68. Mice immunized three times at 3-week intervals with V1-NP DNA (closed circles) or blank vector (open circles) and uninjected controls (open triangles) were challenged 3 weeks after the final immunization. Percent survival is shown for groups of nine or ten mice. Survival of NP DNA–injected mice was significantly greater than controls (P = 0.0004 by chi-square analysis), whereas no significant difference was seen between blank vector–injected mice and uninjected mice (P = 0.17 by chi-square analysis).

by the NP-specific antibodies, as demonstrated by the lack of effect of NP-specific antibodies alone in combating a virus infection, and was thus likely due to NP-specific cellular immunity. Moreover, significant amounts of primary CTLs directed against NP were generated. The protection was against a virulent strain of influenza A that was heterologous to the strain from which the DNA was cloned. Additionally, the challenge strain arose more than three decades after the A/PR/8/34 strain, indicating that immune responses directed against conserved proteins can be effective despite the antigenic shift and drift of the variable envelope proteins. Therefore, the use of an expression vector encoding a viral protein as an immunogen offers a simple means to induce cross-strain protective immunity without the need for self-replicating agents or adjuvants.

In addition, immunization with DNA offers a number of other advantages. First, this approach to vaccination should be applicable to tumors as well as to infectious agents because the $CD8^+$ CTL response is important for both pathophysiological processes (*25*). Therefore, eliciting an immune response against a protein crucial to the transformation process may be an effective means of cancer protection or immunotherapy. Second, the generation of high-titer antibodies to expressed proteins after injection of viral protein (NP and hemagglutinin) and human growth hormone DNA (*26*) indicates that this may be a facile and effective means of making antibody-based vaccines, either separately or in combination with CTL vaccines targeted toward conserved antigens. The ease of producing and purifying DNA constructs compares favorably with traditional protein purification techniques, possibly facilitating the generation of combination vaccines. Finally, because protein expression is maintained after DNA injection (*12*), the persistence of B and T cell memory may be enhanced (*27*), thereby engendering long-lived humoral and cell-mediated immunity.

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10. Comparisons of NP from numerous strains of influenza A have shown no significant differences in secondary structure [M. Gammelin *et al.*, *Virology* **170**, 71 (1989)] and few changes in amino acid sequence [O. T. Gorman *et al.*, *J. Virol.* **65**, 3704 (1991)]. Over an ~50-year period, NP in human strains evolved at a rate of only 0.66 amino acid changes per year. Moreover, our results showing that A/HK/68–specific CTLs recognize target cells pulsed with NP(147–155), derived from the sequence of A/PR/8/34 NP, indicate that this H-$2K^d$–restricted CTL epitope has remained functionally intact over a 34-year span (Fig. 2).
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13. Animals used were female BALB/c mice obtained from Charles River Laboratories, Raleigh, NC. Mice were obtained at 4 to 5 weeks of age and were initially injected with DNA at 5 to 6 weeks of age. Unless otherwise noted, injections of DNA were administered into the quadriceps muscles of both legs, with each leg receiving 50 μl of sterile saline containing 100 μg of DNA. Mice received one, two, or three sets of inoculations at 3-week intervals.
14. The pnRSV-NP (A/PR/8/34) expression vector was made as follows. The NP (A/PR/8/34) gene was isolated from pAPR-501 (*28*) as a 1565-bp Eco RI fragment, was Klenow filled in, and was cloned into the Klenow filled in and phosphatase-treated Xba I site of pRSV-BL. We constructed pRSV-BL by first digesting the pBL-CAT3 vector [B. Luckow and G. Schutz, *Nucleic Acids Res.* **15**, 5490 (1987)] with Xho I and Nco I to remove the chloramphenicol acetyltransferase (CAT) coding sequence and the vector fragment was Klenow filled in and self ligated (designated pBL3). The RSV promoter fragment was isolated as an Nde I and Asp 718 fragment from pRshgrnx [V. Giguere *et al.*, *Nature* **330**, 624 (1987)], Klenow filled in, and cloned into the Hind III site of the intermediate vector generated above (pBL-CAT3 lacking the CAT sequence). The expression vector V1-NP was made as follows. The expression vector V1 was constructed from pCMVIE-AKI-DHFR [Y. Whang *et al.*, *J. Virol.* **61**, 1796 (1987)]. We removed the AKI and DHFR genes by cutting the vector with Eco RI and by allowing the vector fragment to self ligate. This vector does not contain intron A in the CMV promoter, so it was added as a PCR fragment that had a deleted internal Sac I site [at 1855 as numbered in (*29*)]. The template used for the PCR reactions was pCMVintA-Lux, made by ligating the Hind III and Nhe I fragments from pCMV6a120 (*29*), which includes the human CMV IE1 enhancer-promoter and intron A, into the Hind III and Xba I sites of pBL3 to generate pCMVIntBL. The 1881-bp luciferase gene fragment (cut by Hind III and Sma I and Klenow filled in) from RSV-Lux [J. R. de Wet *et al.*, *Mol. Cell. Biol.* **7**, 725 (1987)] was cloned into the Sal I site of pCMVIntBL, which was Klenow filled in and phosphatase treated. The primers that spanned intron A were as follows: 5′-primer, 5′-CTATATAAGCAGAGCTCGTTTAG-3′, and 3′-primer, 5′-GTAGCAAAGATCTAAGGACGGTGACTGCAG-3′. The primers we used to remove the Sac I site were as follows: sense primer, 5′-GTATGTGTCTGAAAATGAGCGTGGAGATTGGGCTCGCAC-3′; and the antisense primer, 5′-GTGCGAGCCCAATCTCCACGCTCATTTTCAGACACATAC-3′. The PCR fragment was cut with Sac I and Bgl II and inserted into the vector, which had been cut with the same enzymes. The NP gene from influenza A (A/PR/8/34) was cut out of pAPR501 (*28*) as above and was inserted into V1 at the blunted Bgl II site to make V1-NP. Plasmids were propagated in *Escherichia coli* and purified by the alkaline lysis method [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989)]. CsCl-banded DNA was ethanol precipitated and resuspended in 0.9% saline to a final concentration of 2 mg/ml for injection.
15. CTLs were generated from mice that had been immunized with DNA or that had recovered from infection with A/HK/68. Control cultures were derived from mice that had been injected with control DNA and from uninjected mice. Single-cell suspensions were prepared, red blood cells were removed by lysis with ammonium chloride, and spleen cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), 0.01 M Hepes (pH 7.5), and 2 mM L-glutamine. An equal number of autologous, irradiated stimulator cells pulsed for 60 min with the H-$2K^d$–restricted peptide epitope NP(147–155) [TYQRTRALV (*30*)] at 10 μM or infected with influenza A/PR/8/34 (H1N1), and recombinant human IL-2 (10 U/ml; Cellular Products, Buffalo, NY) were added, and cultures were maintained for 7 days at 37°C with 5% CO_2 and 100% relative humidity. In selected experiments, recombinant human IL-2 (20 U/ml) and Con A (2 μg/ml) were added in place of autologous stimulator cells. Cytotoxic T cell effector activity was determined with P815 cells labeled for 3 hours with 60 μCi of ^{51}Cr per 10^6 cells and pulsed as above with NP(147–155) or infected with influenza A/Victoria/73 (H3N2). Control targets (labeled P815 cells without peptide or virus) were not lysed. Targets were plated at 1 ×

10^4 cells per well in round-bottomed, 96-well plates and incubated with effectors for 4 hours in triplicate. Supernatant (30 μl) was removed from each well and counted in a Betaplate scintillation counter (LKB-Wallac, Turku, Finland). Maximal counts, released by addition of 6 M HCl, and spontaneous counts released without CTLs were determined for each target preparation. Percent specific lysis was calculated as 100 × (experimental counts − spontaneous counts)/(maximal counts − spontaneous counts).
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20. Female BALB/c mice ≥10 weeks of age were injected intraperitoneally with 0.5 ml of pooled serum [diluted in 2.0 ml of phosphate-buffered saline (PBS)] from mice that had been injected three times with 200 μg of NP DNA. Control mice were injected with an equal volume of pooled normal mouse serum or with pooled serum from mice that had recovered from infection with A/HK/68, also in 2.0 ml of PBS. The dose of A/HK/68 immune serum was adjusted such that the enzyme-linked immunosorbent assay (ELISA) titer of anti-NP antibody was equal to that in the serum pooled from NP DNA–injected mice. Mice were challenged unanesthetized in a blind fashion with 10^4 median tissue culture infectious doses ($TCID_{50}$) of A/HK/68 2 hours after serum injection, and a further injection of an equal amount of serum was given 3 days later. Mice were killed 6 and 7 days after infection, and viral lung titers in $TCID_{50}$ per milliliter were determined as described (*31*).
21. Viral challenges were performed with a mouse-adapted strain of A/HK/68 and maintained subsequently by in vivo passage in mice (I. Mbawuike, personal communication). The viral seed stock used was a homogenate of lungs from infected mice and had an infectivity titer of 5×10^8 $TCID_{50}$/ml on Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, Rockville, MD). For viral lung titer determinations and mass loss studies, viral challenges were performed in blind fashion by intranasal instillation of 20 μl containing 10^4 $TCID_{50}$ on the nares of unanesthetized mice, which leads to progressive infection of the lungs with virus but is not lethal in BALB/c mice (*23*). In survival experiments, mice were challenged by instillation of 20 μl containing $10^{2.5}$ $TCID_{50}$ on the nares under full anesthesia with ketamine and xylazine; infection of anesthetized mice with this dose causes a rapid lung infection that is lethal to 90 to 100% of nonimmunized mice [J. L. Schulman and E. D. Kilbourne, *J. Exp. Med.* **118**, 257 (1963); G. H. Scott and R. J. Sydiskis, *Infect. Immunity* **14**, 696 (1976); (*23*)]. Viral lung titers were determined by tenfold serial titration on MDCK cells in 96-well plates as described (*31*). Data are given as the logarithm of inverse titers.
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26. Neutralizing (hemagglutination inhibiting) antibodies were elicited in mice, ferrets, and rhesus monkeys after injection of DNA encoding influenza A hemagglutinin (*22*). Antibodies to human growth hormone were generated after administration of DNA-coated gold microprojectiles into cells [D.-C. Tang *et al.*, *Nature* **356**, 152 (1992)].
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29. B. S. Chapman *et al.*, *Nucleic Acids Res.* **19**, 3979 (1991).
30. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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32. PCR was performed as per instructions in the GeneAmp kit (Perkin-Elmer Cetus).
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High Levels of HIV-1 in Plasma During All Stages of Infection Determined by Competitive PCR

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Quantitative competitive polymerase chain reaction (QC-PCR) methods were used to quantify virion-associated human immunodeficiency virus type–1 (HIV-1) RNA in plasma from 66 patients with Centers for Disease Control stage I to IVC1 infection. HIV-1 RNA, ranging from 100 to nearly 22,000,000 copies per milliliter of plasma (corresponding to 50 to 11,000,000 virions per milliliter), was readily quantified in all subjects, was significantly associated with disease stage and $CD4^+$ T cell counts, and decreased by as much as 235-fold with resolution of primary infection or institution of antiretroviral therapy. Plasma virus levels determined by QC-PCR correlated with, but exceeded by an average of 60,000-fold, virus titers measured by endpoint dilution culture. Quantitation of HIV-1 in plasma by QC-PCR may be useful in assessing the efficacy of antiretroviral agents, especially in early stage disease when conventional viral markers are often negative.

The natural history of HIV-1 infection is characterized by a variable clinical course, with the development of acquired immunodeficiency syndrome (AIDS) generally occurring after 7 to 11 years (*1*). A central paradox of HIV disease involves the progressive development of immunologic abnormalities, beginning during the early stages of infection when assays for circulating p24 antigen and culturable virus in peripheral blood suggest minimal or absent levels of viral replication (*2–8*). Recent studies of HIV-1 DNA and RNA in clinical samples suggest that, as compared with peripheral blood cells, lymphoid tissue represents a preferred and continuous site of viral replication, although such studies have necessarily been limited by the relative inaccessibility of the tissue compartment, especially for repeated evaluation (*9*).

Previous polymerase chain reaction (PCR) studies of HIV-1 RNA in plasma have generally been limited to qualitative (*10*) or semiquantitative analyses (*11*). In standard PCR methods, the absolute amount of product generated does not always bear a consistent relation to the amount of target sequence present at initiation of the reaction, particularly for clinical specimens (*12*). Both the kinetics and efficiency of amplification of a target template are dependent on the starting abundance of that template and on the sequence match of the primers and target template and may also be affected by inhibitors present in the specimen (*12*). In PCR analysis of RNA samples, variable efficiencies in both the reverse transcription and amplification steps are potential sources of variability. For these reasons, comparison of the amount of specimen-derived PCR product to the amount of product from a separately amplified external control standard (*11*) does not provide a rigorous basis for absolute quantitation. Normalization based on coamplification of a heterologous "internal control" target sequence (such as β-globin or actin) does not optimally address this problem, owing to different template abundances and priming efficiencies for different primer-target combinations.

In the quantitative competitive PCR (QC-PCR) method for RNA quantitation (*13*) a competitive RNA template matched to the target sequence of interest, but differing from it by virtue of an introduced internal deletion, is used in a competitive titration of the reverse transcription and PCR steps, providing stringent internal control (*13–15*). Increasing known copy numbers of competitive template are added to replicate portions of the test specimen, and quantitation is based on determination of the relative, not absolute, amounts of the

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