Increased Expression and Immunogenicity of Sequence-Modified Human Immunodeficiency Virus Type 1 gag Gene

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A major challenge for the next generation of human immunodeficiency virus (HIV) vaccines is the induction of potent, broad, and durable cellular immune responses. The structural protein Gag is highly conserved among the HIV type 1 (HIV-1) gene products and is believed to be an important target for the host cell-mediated immune control of the virus during natural infection. Expression of Gag proteins for vaccines has been hampered by the fact that its expression is dependent on the HIV Rev protein and the Rev-responsive element, the latter located on the env transcript. Moreover, the HIV genome employs suboptimal codon usage, which further contributes to the low expression efficiency of viral proteins. In order to achieve high-level Rev-independent expression of the Gag protein, the sequences encoding HIV-1SF2 p55Gag were modified extensively. First, the viral codons were changed to conform to the codon usage of highly expressed human genes, and second, the residual inhibitory sequences were removed. The resulting modified gag gene showed increases in p55Gag protein expression to levels that ranged from 322- to 966-fold greater than that for the native gene after transient expression of 293 cells. Additional constructs that contained the modified gag in combination with modified protease coding sequences were made, and these showed high-level Rev-independent expression of p55Gag and its cleavage products. Density gradient analysis and electron microscopy further demonstrated that the modified gag and gagprotease genes efficiently expressed particles with the density and morphology expected for HIV virus-like particles. Mice immunized with DNA plasmids containing the modified gag showed Gag-specific antibody and CD8+ cytotoxic T-lymphocyte (CTL) responses that were inducible at doses of input DNA 100-fold lower than those associated with plasmids containing the native gag gene. Most importantly, four of four rhesus monkeys that received two or three immunizations with modified gag plasmid DNA demonstrated substantial Gag-specific CTL responses. These results highlight the useful application of modified gag expression cassettes for increasing the potency of DNA and other gene delivery vaccine approaches against HIV.

The induction of long-lasting, potent humoral and cellular immune responses will be important for an effective human immunodeficiency virus (HIV) vaccine. Data from HIV-infected patients, and in particular from long-term nonprogressors, have shown that viral structural genes can elicit substantial immune responses. Gag-specific CD8+ cytotoxic T lymphocytes (CTL) have been shown to be important in controlling virus load during acute infection (4, 21) as well as during the asymptomatic stages of the infection (20, 24). Moreover, a strong Gag-specific CTL response appears to correlate inversely with the viral load of HIV-1-infected patients (7). In addition, studies of exposed but uninfected prostitutes indicate that Gag-specific CTL may be involved in protection against the establishment of a persistent HIV type 1 (HIV-1) infection (28). Combined, these studies provide convincing evidence that immune responses directed against HIV Gag proteins may be an important component of an effective HIV vaccine. The usefulness of Gag immunogens for vaccines is further indicated by the fact that the protein is relatively conserved among diverse HIV strains and subtypes, and cross-clade CTL recognition directed against Gag-specific targets has been well documented (2, 3, 11, 23).

Immunization with naked DNA or recombinant virus induces both antibody and CTL responses and has been shown to be an efficient method of eliciting protective immune responses against a broad range of pathogens in animal studies (10). However, the potency of current gene delivery methods such as naked-DNA and viral vectors must be improved to induce adequately robust responses for protection in primates (1). One means to achieve this may be through increasing the expression efficiency of encoded HIV antigens. The poor expression of the HIV structural genes in recombinant vectors is caused by a strong Rev dependency that allows efficient expression only in the presence of the viral Rev protein (25, 30). The translation efficiency and stability of gag transcripts are further decreased by the presence of a relatively high AU content and destabilizing AUUUA motifs (inhibitory sequences [INS]). In previous studies, inactivation of these INS enabled the Rev-independent expression of HIV-1 gag (29), but these modifications reduced the approximate AT content of the gag gene only from 56 to 50%. Elevated percentages of AU in human mRNAs have been shown to result in instability, increased turnover, and low expression levels (15). These findings suggest that further reductions of the AT content of the gag gene could result in improved mRNA stability and increased protein expression. In support of this, it has been shown that highly expressed human genes employ codon usage patterns different from those used by HIV genomes. For highly expressed genes, G or C is generally preferred over A or T. Furthermore, changes in the codon usage of HIV-1 env to those employed by highly expressed human codons resulted in increased Rev-independent expression (14).

In order to achieve high-level Rev-independent expression
of the gag gene of HIV-1gp24, the codon usage pattern was first altered to conform to that used by highly expressed human genes (14). Further modifications were then made to remove possible residual INS motifs previously identified in the gag coding region (29). This resulted in lowering the AT content of the gag coding sequences from 56 to 32%, a level more consistent with increased mRNA stability and translation efficiency. The sequence-modified HIV-1SF2 gag gene was inserted into a high-level plasmid expression vector for in vitro transfections and DNA immunization studies with rodents and nonhuman primates. Results presented here indicate that sequence-modified gag plasmids expressed protein at dramatically higher levels and showed increased immunogenicity compared to the native gag sequence in DNA immunization experiments performed with mice and rhesus macaques. Additionally, the inclusion of modified protease-coding sequences in the modified gag resulted in high-level Rev-independent expression, processing of the Gagprotease polyprotein, and the production of virus-like particles (VLP) with the morphologies of both immature and mature HIV-1 virions.

MATERIALS AND METHODS

gag and gagprotease plasmids. The native sequences coding for the 502 amino acids (aa) of HIV-1gp24 p55Gag (GenBank accession no. K02007) (14) were further inactivated without altering the reading frame for the p55Gag nucleic acid sequence-modified gag plasmids expressed protein at dramatically higher levels and showed increased immunogenicity compared to the native gag sequence in DNA immunization experiments performed with mice and rhesus macaques. Additionally, the inclusion of modified protease-coding sequences in the modified gag resulted in high-level Rev-independent expression, processing of the Gagprotease polyprotein, and the production of virus-like particles (VLP) with the morphologies of both immature and mature HIV-1 virions.

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Na₂ suspensions were prepared by treatment with Tris-buffered NH₄Cl (Sigma). Erythrocyte-depleted single cell gag-pol were taken 5 days post-rVV challenge. Erythrocyte-depleted single cell gag-pol were transferred to Lumaplates (Packard, Gaithersburg, Md.), dried, and counted in a Microbeta scintillation counter (Wallac, Gaithersburg, Md.). Percent specific ⁵¹Cr release was determined from the formula percent specific ⁵¹Cr release = (mean experimental release – mean spontaneous release)/(maximum release – spontaneous release) × 100%, where spontaneous release = mean counts per minute released from target cells in the absence of spleen cells and maximum release = mean counts per minute released from target cells in the presence of 0.1% Triton X-100.

Measurement of Gag-specific IFN-γ-producing CD8 lymphocytes. Splenocytes were taken 5 days post-rVgag-pol challenge. Erythrocyte-depleted single cell suspensions were prepared by treatment with Tris-buffered NH₄Cl (Sigma). Nucleated spleen cells (1 × 10⁶ to 2 × 10⁶) were cultured in duplicate at 37°C in the presence or absence of 10 μg of rVgag/ml. Monensin (Pharmingen, San Diego, Calif.) was added to block cytokine secretion. After 3 to 5 h cells were washed, incubated with anti-CD16/32 (Pharmingen) to block Fc receptors, and fixed in 2% (wt/vol) paraformaldehyde and stored overnight at 4°C. The following day cells were treated with 0.5% (wt/vol) saponin (Sigma) and stained with a phycoerythrin (Pharmingen)-conjugated mouse IFN-γ MAb in the presence of 0.1% (wt/vol) saponin. Cells were then washed free of saponin, stained with fluorescein isothiocyanate-conjugated CD8 MAb (Pharmingen), washed, and then analyzed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems). Samples were cultured and stained in duplicate.

Peptide pools. A set of 51 Gag peptides 20 residues long, overlapping by 10 aa and spanning residues 1 to 496 of HIV-1SF2 p55Gag, was synthesized (Chiron Mimotopes, Clayton, Australia). Eight pools were made by mixing 5 to 7 overlapping peptides. Gag amino acid sequences spanned by the pools are as follows: aa 1 to 80, pool 1; aa 71 to 144, pool 2; aa 135 to 203, pool 3; aa 194 to 263, pool 4; aa 254 to 323, pool 5; aa 314 to 365, pool 6; aa 351 to 430, pool 7; aa 421 to 496, pool 8. A pool of six 20-aa overlapping peptides representing HIV-1SF2 Env served as a negative-control pool.

Purification of rhesus macaque PBMC and derivation of B-LCL. Rhesus macaque peripheral blood mononuclear cells (PBMC) were separated from heparinized whole blood on Percoll gradients and cultured at 3 × 10⁶ to 3.5 × 10⁷ per well in 1.5 ml in 24-well plates for 8 days in AIM-V–RPMI 1640 (50% culture medium; Gibco) supplemented with 10% FCS. Gag-specific cells were stimulated by the addition of either a Gag peptide pool (13.3 μg of total peptide/ml) or autologous PBMC that had been infected with rVgag-pol and cultured in 24-well plates. Recombinant human interleukin-2 (IL-2; 15 ng/ml; R&D Systems, Minneapolis, Minn.) was added at the initiation of culture. Human recombinant IL-2 (20 IU/ml; Proleukin; Chiron) was added on days 1, 3, and 6. For the derivation of stable rhesus B-lymphoblastoid cell lines (B-LCL), PBMC were exposed to herpesvirus papio-containing culture supernatant from the S594 cell line (13, 27) in the presence of 1 μg of cyclospermine (Sigma)/ml.

Rhesus macaque CTL assay. Autologous B-LCL were labeled overnight with Na₂⁴ⁱ¹⁵CrO₄ (NEN, Boston, Mass.; 25 μCi per 10⁶ B-LCL) and washed. Individual aliquots were then incubated for 1 h with 100 μg of Gag or Env peptide pool/ml (Peptide-pulsed. ⁵¹Cr-labeled B-LCL were added (2,500 per round-bottom well) to duplicate wells containing threefold serial dilutions of cultured PBMC. Unlabeled B cells (10⁷) were added to each well to inhibit nonspecific cytolytic activity. After 4 h, 50 μl of culture supernatant was harvested, added to Lumaplates (Packard), and counted with a Microbeta 1450 liquid scintillation counter (Wallac). ⁵¹Cr released from lysed targets was normalized by the formula percent specific ⁵¹Cr release = 100% × (mean experimental release – mean spontaneous release)/maximum release – spontaneous release, where spontaneous release = mean counts per minute released from target cells in the absence of spleen cells and maximum release = mean counts per minute released from target cells in the presence of 0.1% Triton X-100.

RESULTS

Increased in vitro expression efficiency of sequence-modified HIV-1SF2 gag gene. The coding sequences for the HIV-1SF2 gag gene were modified to conform to the codon usage pattern of highly expressed human genes and to eliminate residual INS motifs as described in Materials and Methods. These modifications resulted in gag coding sequences with a clear reduction in overall AT content compared to that of the native gag (Fig. 1). In fact, the percentage of A and T nucleotides was reduced from 56 to 32%, a level more consistent with increased mRNA stability and translation efficiency (14, 15). The AT content of the modified gag more closely resembled that of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, which encodes a relatively stable mRNA compared with the relatively unstable AU-rich human IFN-γ mRNA (Fig. 1).

The in vitro expression efficiency of the modified HIV-1SF2 gag (pCMVkm2.GagMod.SF2) was compared to that of the native SF2 gag in a construct (pCMVLink.Gag.SF2.PRE) that also contained the hepatitis B virus PRE. The pCMVLink.Gag.SF2.PRE plasmid previously had been found to express Gag at substantially higher levels than a similar plasmid containing the HIV-1SF2-derived gag gene without the PRE (S. W. Barnett, unpublished data). The expression levels for these plasmids were determined in several independent experiments after transfection of three different cell lines, RD, 293, and COS-7 (Table 1). Cell supernatants and lysates were tested at 48 and 60 h posttransfection. Gag expression levels were clearly much higher for the modified gag plasmid at all time points and in all three cell lines tested. The increased expression was most dramatic in the supernatants of the transfected human 293 cell line, where expression from the modified gag...
As shown in Fig. 3A, budding and free immature particles 24 h posttransfection and electron microscopy was performed. Transfected with pCMVKm2.GagMod.SF2 were harvested at 60 h posttransfection and centrifuged through 20 to 60% sucrose density gradients. Gradient fractions were collected, run on an SDS–8% polyacrylamide gel, and analyzed by Western blotting as described in Materials and Methods. (A) Immunoblot of fractions 1 to 9 from the sucrose density gradient from transfection supernatants of the modified gag plasmid. (B) Immunoblot comparing peak fractions collected in the density range expected for HIV-1 VLP after transfection with modified (Mod) or native (Nat) HIV-1SF2.gag plasmids. Vector alone (Neg) was used as a negative transfection control, and the prestained broad-range molecular weight marker (M) was used as the size standard.

**TABLE 1. Increased in vitro expression from modified versus native gag plasmids in supernatants and lysates from transiently transfected cells**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Type of plasmid</th>
<th>Material assayed</th>
<th>Cell line</th>
<th>Hours post-transfection</th>
<th>Total ng p24 (fold increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nat</td>
<td>Sup</td>
<td>293</td>
<td>48</td>
<td>3.4</td>
</tr>
<tr>
<td>2</td>
<td>Mod</td>
<td>Sup</td>
<td>293</td>
<td>48</td>
<td>1,260 (371)</td>
</tr>
<tr>
<td>3</td>
<td>Nat</td>
<td>Sup</td>
<td>293</td>
<td>60</td>
<td>3.2</td>
</tr>
<tr>
<td>4</td>
<td>Mod</td>
<td>Sup</td>
<td>293</td>
<td>60</td>
<td>2,222 (694)</td>
</tr>
<tr>
<td>5</td>
<td>Nat</td>
<td>Sup</td>
<td>293</td>
<td>60</td>
<td>1.8</td>
</tr>
<tr>
<td>6</td>
<td>Mod</td>
<td>Sup</td>
<td>293</td>
<td>60</td>
<td>1,740 (966)</td>
</tr>
<tr>
<td>7</td>
<td>Nat</td>
<td>Sup</td>
<td>293</td>
<td>60</td>
<td>1.5</td>
</tr>
<tr>
<td>8</td>
<td>Mod</td>
<td>Sup</td>
<td>293</td>
<td>60</td>
<td>580 (322)</td>
</tr>
<tr>
<td>9</td>
<td>Nat</td>
<td>Lys</td>
<td>293</td>
<td>60</td>
<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td>Mod</td>
<td>Lys</td>
<td>293</td>
<td>60</td>
<td>85 (57)</td>
</tr>
</tbody>
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**FIG. 2.** Increased expression in vitro of HIV-1SF2.p55Gag particles in cells transfected with the modified gag gene. 293 cells were transfected with plasmids containing either the modified or native HIV-1SF2 gag genes. Supernatants from transfected cell cultures were collected at 60 h posttransfection and centrifuged through 20 to 60% sucrose density gradients. Gradient fractions were collected, run on an SDS–8% polyacrylamide gel, and analyzed by Western blotting (Fig. 2A) to determine the peak fraction of each sample. Western blot analysis showed that the p55 Gag band for the pCMVLink.Gag.SF2.PRE plasmid was 322- to 966-fold greater than that of the native HIV-1SF2 gag plasmid tested. The improvement in Gag expression levels in 293 cell lysates was also apparent, but less so than in the supernatants, which could be indicative of more-efficient budding of p55Gag particles in cells where expression levels are elevated. To exclude possible effects on the transfection efficiency depending on the plasmid used, flow cytometry and direct fluorescence microscope analysis were done in parallel transfections or by cotransfection using GFP plasmid DNA. On average, 70% of the cells were transfected using either transfections or by cotransfection using GFP plasmid DNA. Western blotting (Fig. 2A) to determine the peak fraction of each sample. Western blot analysis showed that the p55 Gag band for the modified Gag expression cassette was stronger than that for the best native gag plasmid (Fig. 2B).

To confirm that VLP were being expressed, COS-7 cells transfected with pCMVKm2.GagMod.SF2 were harvested at 24 h posttransfection and electron microscopy was performed. As shown in Fig. 3A, budding and free immature particles could be observed. These data confirm that the sequence modifications for the gag gene did not adversely affect the p55Gag particle assembly or VLP morphology.

**Construction and characterization of sequence-modified gagprotease gene cassettes.** As a first step in the design of modified HIV immunogens with increased representation of Pol-specific epitopes, two different modified gagprotease gene constructs were evaluated for expression and VLP formation. The protease coding sequences in these constructs were (i) codon optimized, with subsequent INS inactivation as described above for gag (GP1), or (ii) modified by INS inactivation alone (GP2). Like the modified gag plasmid, in the absence of Rev both versions of the modified gagprotease exhibited high-level expression of Gag proteins in supernatants and cell lysates of transiently transfected COS-7 and 293 cell lines (Table 2). In fact, the expression levels measured in lysates of 293 cells transfected with the gagprotease plasmids were higher than those seen with the modified gag alone. This result could be partially or wholly attributed to more-efficient recognition of processed Gag (mostly p24) than of unprocessed p55Gag by the Coulter p24 antigen capture assay, as has been previously described (29). This apparent increase in p24 expression in cell lysates was not observed in COS-7 cells, possibly due to lower overall expression of p55Gag in this cell line. Sucrose density gradient analyses of supernatants from 293 and COS-7 cells transiently transfected with either gagprotease or gag constructs were performed, and the peak fractions were subsequently analyzed by Western blotting. The efficiency of VLP formation varied between the cell lines tested and was found to be lower for gagprotease than for the modified gag plasmid (Fig. 4). The levels of VLP formation from the two gagprotease constructs in 293 cells were similar (Fig. 4A; GP1 and GP2), but the analysis of the codon-optimized and INS-inactivated gagprotease plasmid, GP1, in COS-7 cells suggested the production of relatively small amounts of VLP (Fig. 4B). Polypeptides expressed from both of the modified versions of gagprotease were correctly processed by the encoded viral protease. Bands corresponding to unprocessed p55Gag and completely processed p24 were detectable using a MAb specific for p24 (data not shown) or HIV-1+ patient serum (Fig. 4A and B) (p17 levels were too low to be detected with the HIV+ sera used).
Electron microscopic analysis of COS-7 cells transfected with the two different sequence-modified gagprotease constructs confirmed the results of the sucrose gradient analysis. COS-7 cells transfected with the codon-optimized and INS-inactivated version (GP1) showed very little VLP formation (data not shown) compared to those transfected with the non-optimized INS-inactivated gagprotease (GP2; Fig. 3B). A possible explanation for this observation is that codon optimization of the protease coding sequences may have resulted in its overexpression relative to Gag and the prevention of the efficient budding of particles (19). The GP2 version of gagprotease, in which the INS of the protease-coding region was inactivated without codon optimization, reduced protease overexpression, and thus VLP of the mature and immature phenotypes could be detected.

**Increased immunogenicity of the modified gag DNA in vivo.**
To evaluate and compare the immunogenicities of the modified and the native gag plasmids, mice were immunized intra-muscularly with plasmid DNA doses titrated from 20 to 0.02 μg per mouse. Serum was collected at 4 weeks postimmunization and tested in a p24 gag-specific antibody ELISA. Antibody responses to Gag could be detected in mice immunized with as little as 0.2 μg of the modified gag expression cassette, whereas the native gag cassette was able to induce an antibody response only at the 20-μg DNA dose (Fig. 5A). This represented the induction of an antibody response using the modified gag at a single DNA dose 100 fold lower than that necessary for the native gag. In parallel groups of animals, a second dose of DNA was given at 4 weeks to determine if antibody responses to the modified gag had reached maximal values at the 20-μg dose and if the lowest DNA dose of 0.02 μg could induce an antibody response after a second immunization. As shown in Fig. 5B, the Gag-specific antibody titers increased after the second immunizations for all DNA doses except for the 0.02-μg DNA dose group, which remained negative.

Measurements of the cellular immune responses following DNA immunization with the modified gag demonstrated a similar pattern. Gag-specific CTL responses were inducible at DNA amounts at least 10-fold lower than those necessary with...
the native gag expression cassette (Fig. 6). Gag-specific CTL were detectable after a single immunization with a dose of the modified gag plasmid DNA as low as 0.02 µg, whereas a dose of 0.2 µg of the native gag plasmid was required for the induction of detectable CTL. In a subsequent study, the modified gag plasmid DNA was further diluted (down to 0.2 ng) and used to immunize additional groups of mice. As shown in Fig. 7, Gag-specific IFN-γ-positive CD8+ T cells were scored in mice receiving as little as 2 ng of the modified gag DNA.

**Induction of CTL responses in rhesus macaques immunized with the modified gag plasmid.** Based on the increased potency observed in mouse immunizations with the modified gag plasmid DNA, studies with nonhuman primates were initiated. Four rhesus macaques were given three intramuscular immunizations with 1-mg doses of gag plasmid at 4-week intervals. PBMC were harvested prior to immunization and at 2 weeks after the second and third immunizations. PBMC were cultured with Gag peptide pools or with rVVgag-pol-infected autologous PBMC to stimulate the expansion and differentiation of CTL and tested against Gag peptide pool-pulsed. 51Cr-labeled, autologous B-LCL targets in 4-h 51Cr release assays. No Gag-specific cytolysis in PBMC was observed prior to immunization (not shown). However, after gag DNA immunization, all four macaques showed cytolytic activity against autologous B-LCL pulsed with at least one Gag peptide pool. In addition, two of the four macaques reacted with two or three Gag peptide pools (Fig. 8). Percent specific lysis of Gag-pulsed target cells varied among animals and among pools and reached as high as 80% at the highest effector cell/target cell ratio (Fig. 8C). A Gag-specific antibody response (antibody titer, 164) was detected in one of the four animals 2 weeks after the second immunization. This animal also had an anamnestic immune response 2 weeks after the third immunization, with a fivefold increase of the antibody titer (890). A second animal

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**FIG. 5.** Increased immunogenicity of the modified HIV-1 gag plasmid compared to that of the native gag plasmid. Groups of mice were immunized bilaterally in the tibialis anterior muscles with titrated amounts of DNA in 10-fold dilutions from 20 µg down to 0.002 µg. Sera were collected at 0 and 4 weeks and tested for HIV-1 p24-specific antibody titers by ELISA as described in Materials and Methods. (A) Comparison of humoral immune responses at different DNA doses using the native and modified gag plasmid DNA. Values represent the geometric mean antibody titers and the standard deviations of the midpoint antibody titers for each group. The values in parentheses indicate the percentages of responders (percent seroconversion) in each group. (B) Antibody responses were boosted following a second immunization with the modified gag plasmid DNA. Four weeks after the first immunization, additional groups of mice received a second immunization with the same amount of titrated plasmid DNA. Sera collected at weeks 0, 4, and 6 were analyzed by p24 antibody ELISA.

**FIG. 6.** CTL responses in CB6F1 mice after a single immunization with titrated plasmid DNA. Nine weeks after immunization mice were challenged with an intraperitoneal dose of 106 PFU of rVVgag-pol. Five days later effector (E) spleen cells were tested for cytolytic activity in a 4-h 51Cr release assay using 51Cr-labeled SVB/ALB tumor target (T) cells (5,000 targets per well) that had been pulsed for 1 h with a 1-µg/ml concentration of the H-2Kd-binding HIV-1 Gag peptide p7g (●). Target cells pulsed with the negative-control HIV-1 Gag peptide pgagb (○) and major histocompatibility complex-mismatched (H-2b), p7g peptide-pulsed RMA target cells (△) were employed as negative controls.

**FIG. 7.** Quantification of Gag-specific, IFN-γ-producing CD8+ T lymphocytes in mice after a single immunization of titrated modified gag plasmid DNA followed by rVVgag-pol challenge. Spleenic IFN-γ-positive CD8+ T lymphocytes specific for the p7g Gag peptide were enumerated by flow cytometry as described in Materials and Methods. mock, results using spleen cells from naive mice.
had a very low titer 2 weeks after the second immunization (65), which later dropped below the detection level. These results reflect the induction of robust and relatively broad CTL responses. A single dose of 200 ng of the modified gag was sufficient to induce measurable anti-Gag antibody responses in 25% of the mice (Fig. 1A), while 100-fold more (20 μg) of the native gag plasmid was required for the detection of Gag-specific antibodies.

The improved potency of the codon-modified gag expression plasmid observed in mouse studies was confirmed with rhesus macaques. Four of four macaques had detectable Gag-specific CTL after two or three 1-mg doses of modified gag plasmid. In contrast, in a previous study, only one of four macaques given 1-mg doses of plasmid DNA encoding the wild-type HIV-1SF2 Gag showed strong CTL activity, which was not apparent until after the seventh immunization (X. Paliard and C. Walker, unpublished data). Further evidence of the potency of the modified gag plasmid was the observation that CTL from two of the four rhesus macaques reacted with three nonoverlapping Gag peptide pools, suggesting that as many as three different Gag peptides are recognized and indicating that the CTL response is polyclonal. Additional quantification and specificity studies are in progress to further characterize the T-cell responses to Gag in plasmid-immunized rhesus macaques. DNA immunization of macaques with the modified gag plasmid did not result in significant antibody responses, with only two of four animals seroconverting at low titers. In contrast, the majority of macaques in additional groups immunized with p55Gag protein seroconverted and had strong Gag-specific antibody titers (G. Otten, unpublished data). These preliminary data together with data from other investigators indicate that a prime-boost strategy, with DNA prime and protein boost, could be very promising for the induction of strong CTL and antibody responses.

These results indicate that sequence-modified high-level expression cassettes for HIV structural genes can improve the potency of plasmid-vectorized HIV vaccines. Sequence-modified genes may also enhance the potency of virus-vectorized vaccines and increase the production efficiency of HIV structural proteins for use in subunit vaccines.

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