Envelopes of primary R5-tropic human immunodeficiency virus type 1 (HIV-1) isolates may be particularly relevant for vaccine purposes and should be evaluated for immunogenicity in animals including macaques before carrying out human vaccine trials. In the present study, the immunogenicities of synthetic HIV-1 env DNA vaccines, which had been derived from the early primary isolate Bx08 and contain humanized codons, were evaluated in mice, guinea pigs and rhesus macaques. Neutralization sensitivity of the HIV-1 Bx08 isolate was found to resemble that of other primary isolate prototypes. Immunogenicity of gp120 delivered as codon-optimized DNA vaccine was comparable to that of recombinant gp120 protein plus adjuvant in mice. Similarly, DNA vaccination of guinea pigs with synthetic gp140 Bx08 and gp150 Bx08 DNA induced a strong antibody response independent of the gene construct and DNA immunization route. Mamu-A*01 rhesus macaques were DNA vaccinated with synthetic gp150 Bx08 or gp140 Bx08 DNA and boosted with a replication-deficient recombinant human adenovirus type 5 expressing a synthetic gp120 Bx08 gene. DNA-vaccinated rhesus macaques developed specific CD8+ T lymphocyte responses and anti-rgp120 IIIb antibody responses. Both the humoral and cellular responses were significantly improved following intramuscular boosting with the recombinant adenovirus. The demonstrated humoral and cellular immunogenicities of these HIV Bx08 Env vaccines in non-human primates encourages their further development as one component in candidate HIV vaccines for humans.
So far, there is only one ongoing trial that includes an Env from primary R5-tropic isolates (Francis et al., 1998; Ruxrungham & Phanuphak, 2001). Primary isolates are less sensitive than T cell line-adapted (TCLA) HIV-1 isolates to neutralization by soluble CD4 (sCD4), monoclonal antibodies, and patients’ immune sera (Beddows et al., 1998; Bures et al., 2000; Follis et al., 1998; Moore et al., 1993; Wrin et al., 1995; Zhang et al., 1997). Furthermore, primary isolates and TCLA virus use different co-receptors, principally indicating distinct properties of the envelope glycoproteins (Zhang et al., 1996). A relevant envelope subunit vaccine candidate should represent the circulating HIV-1 strains and preferably contain neutralizing antibody epitopes common to heterologous isolates. Therefore, we derived envelope genes from the R5-tropic strain HIV-1Bx08, isolated early in infection (Moog et al., 1997a, b).

Immunization studies using recombinant gp120 have shown very limited success in generating functional neutralizing antibodies in humans (Connor et al., 1998; Graham et al., 1998; Mascola et al., 1996). It has also been shown that the main part of the antibody response seen in natural infection is directed against gp120 viral debris and has little effect against native envelope proteins (Parren et al., 1997). DNA vaccines that express the antigens in situ and thus mimic native Env proteins have induced both antibodies and cytotoxic T lymphocytes (CTL) and provided a degree of protection against pathogenic virus challenge (Egan et al., 2000; Lu et al., 1996). Strong antibody responses and CTL has been shown to delay disease progression following SIVmac251 or SHIV infection (Buge et al., 1999; Shiver et al., 2002). Despite these achievements, no vaccine study has yet reported induction of complete protection against challenge with heterologous primary HIV-1 or SHIV isolates.

We and others have shown that complete humanization of codons of DNA-encoded HIV antigens in combination with an efficient signal peptide sequence and a strong promoter in the plasmid improves the priming (André et al., 1998; Corbet et al., 2000; Haas et al., 1996; Shiver et al., 2002; Vinner et al., 1999). For optimal boosting of DNA-primed immune responses a number of viral vectors hold great potential. Among these, replication-deficient adeno-virus has proved to be effective in boosting cellular and humoral response (Shiver et al., 2002). Here, we explore the potential of a vaccination regime consisting of intramuscular (i.m.) priming with high doses of codon-optimized second-generation DNA vaccines followed by boosting with a recominant replication-deficient adenovirus type 5 (rAd5) expressing env from primary HIV-1Bx08. This vaccination regime aims to prime both humoral and cellular immune responses. These properties would appear to be particularly attractive for vaccine development against HIV-1 for which immune control may depend on a combination of neutralizing antibodies and T cell-mediated immune responses. To our knowledge, this is the first immunization study conducted in non-human primates using completely humanized HIV-1 envelope genes derived from a vaccine relevant primary isolate.

**METHODS**

**Plasmids.** We have used the synthetic Bx08 envelope gene derivative from a primary R5-tropic isolate. As described earlier the HIV-1Bx08 was isolated from an infected individual 8 months after seroconversion (Moog et al., 1997a). The genes were designed with wild-type amino acid sequence but exclusively reflecting the codon-usage of highly expressed human genes. Briefly, the genes were assembled from long synthetic oligonucleotides that were individually cloned (Corbet et al., 2000). To obtain the secreted gp140 and the membrane-bound gp150 gene products stop codons were introduced before or after the transmembrane region. The envelope constructs both contain the intact gp120–gp41 cleavage site and the Mamu-A*01-restricted HIV-1 envelope CTL epitope YAPPIGGQI (Egan et al., 1999). The constructed genes were cloned in the WRG0709 plasmid (Powderject, Madison, WI, USA) with the following key functional elements for eukaryotic expression: the CMV-IE promoter, intron-A and the tPA secretory signal.

Plasmid DNA for inoculation in mice and guinea pigs was purified using the Qiagen Endofree Plasmid Mega Kit. Large-scale plasmid DNA preparations for inoculation in macaques were performed under GMP conditions by GeneCare (Lyngby, Denmark). The endotoxin content of these preparations was <5 EU mg⁻¹.

**Recombinant adenovirus.** HIV-gp120 cDNA fragment was cloned into a transfer plasmid first and then subcloned into an E1- and E3-deleted adeno-virus plasmid through a convenient green/white selection process (Farina et al., 2001). The molecular clone of pAdCMVHIV-gp120 was transfected into 293 cells for virus rescue. Once the recombinant virus was rescued and expanded to a large-scale infection, virus purification was performed using the CsCl sedimentation method (Gao et al., 1996). Before its use for animal experiments, the purified virus preparation was characterized for genome structure, infectivity expressed as p.f.u., transgene expression in vitro and replication competent adeno-virus (RCA). The preparation was RCA free at the level of sensitivity of the assay (1 RCA per 10⁷ p.f.u. of the test virus).

**Inoculation of animals.** Seven- to eight-week-old BALB/c mice (H-2¹) purchased at Bomholtgaard (Denmark) and AL guinea pigs bred at Statens Serum Institut (SSI) (Allerød, Denmark) were housed at SSI’s Animal Facility. In all animals microbiological status was conventional and the acclimatization period before initial immunization was at least 1 week. Blood samples were taken at 2- or 3-week intervals. I.m. injections of five mice with syn.gp120Bx08 plasmid DNA were administered in the tibia anterior muscle at weeks 0, 9 and 15 using 2× 50 μl of 2 μg DNA ml⁻¹ in endotoxin-tested PBS. BALB/c mice were injected i.m. at weeks 0, 2, 4 and 12 with 2 μg per immunization of recombinant gp120gp1 plasmid DNA in 10 μg of QuadriA adjuvant (Kamstrup et al., 2000). I.m. injections of guinea pigs were administered as described for mice at weeks 0, 9 and 24. For comparison, guinea pigs were also inoculated intradermally (i.d.) with a gene gun at weeks 0, 3, 6, 9 and 24 on shaved abdominal skin with plasmid DNA-coated gold particles using the Helios gene gun (Bio-Rad). Each animal was shot four times per immunization with gold pellets containing 0-5 mg gold per shot with 2 μg plasmid DNA (mg gold)⁻¹. DNA bullet coating efficiency was >60 %.

Six rhesus macaques (Macaca mulatta) positive for the Mamu-A*01 major histocompatibility complex class I (MHC-I) were bred and immunized at MRC (Oxford, UK) in accordance with the guidelines of the Home Office Code of Practice. All procedures were carried...
out under general anaesthesia. The macaques received four i.m. needle injections with 5 mg of DNA in 2-5 ml PBS in an initial priming series of three immunizations (weeks 0, 4 and 8) plus one DNA booster immunization at week 24. Two macaques, Gaffa and Gilda, were vaccinated with syn.gp150Bx08 plasmid DNA and subsequently boosted i.m. with 4×10^{11} p.f.u. of recombinant syn.gp120Bx08 adenovirus at week 48. An additional two macaques, Gloria and Gege, were vaccinated with syn.gp140Bx08 plasmid DNA and boosted with 4×10^{11} p.f.u. of recombinant syn.gp120Bx08 adenovirus at week 32. The two control animals, Jolly and Jez, were vaccinated once with recombinant syn.gp120Bx08 adenovirus only.

**Anti-rgp120 IgG antibody ELISA.** IgG antibodies to recombinant gp120Bx08 were measured in indirect ELISA as described earlier (Corbet et al., 2000). For analysis of macaque samples HRP/rabbit anti-human IgG (Dako) was used. The conjugate antibodies used for analysis of mice and guinea pig samples were HRP/rabbit anti-mouse (Dako) and HRP/rabbit anti-guinea pig IgG (Sigma), respectively. All anti-rgp120Bx08 antibody titres were expressed as the reciprocal sample dilution, giving an absorbance (A) value (492 nm) of 0-500, which represent the steepest slope, using A_{50} as reference wavelength. The titres were expressed relative to serially diluted standard sera with calibration control samples included on all plates. In addition, sera were tested in two or more dilutions to cover a wide concentration range and at the same time to confirm parallelism with the standard.

**Antibody displacement ELISA.** To measure the relative avidity of the macaque antisera an antibody displacement ELISA was performed as described for rgp120Bx08. ELISA was with an additional 15 min incubation before addition of conjugate antibody using 200 μl of urea adjusted to 0, 2, 4, 6 or 8 M in PBS followed by five washes in wash buffer. Prior to the assay, samples were adjusted to similar concentrations of rgp120Bx08-specific IgG. All measurements gave absorbance values in the linear range of the assay, decreasing with increasing urea concentration. The results were normalized and expressed as the absorbance at the half-maximal urea concentration (M) relative to the absorbance without urea.

**Neutralization assay.** Neutralization of the primary HIV-1Bx08 isolate was measured in phytohaemagglutinin-P-stimulated peripheral blood mononuclear cells (PBMC) essentially as described elsewhere (Zhou & Montefiori, 1997). Briefly, triplicates of serial dilutions of heat-inactivated plasma and serum samples were incubated for 1 h at 37 °C with 50 TCID_{50} ml^{-1} of HIV-1Bx08 virus. Subsequent infections of 1 × 10^{5} PBMCs per well were terminated after 4 h or overnight incubation for plasma and serum samples respectively. Culture supernatants were harvested daily. The harvest from the day at which p24 production was in an early linear phase of increase was used for measurement of the p24 concentration in all samples with an in-house p24 antigen ELISA (Nielsen et al., 1987; Zhou & Montefiori, 1997). Characterization of the neutralization sensitivity of the HIV-1Bx08 isolate was performed with a panel of antisera from infected individuals, which has been described elsewhere (Bures et al., 2000). In addition, samples from Danish patients were included. The inhibitory concentration (IC_{50}) was calculated by interpolation and expressed as the reciprocal dilution of the sample giving an 80 % reduction in p24 antigen. A known positive control serum sample was included on each plate to ensure reproducibility.

**ELISPOT assay.** Cellular IFN-γ release upon specific peptide restimulation was assessed in an ELISPOT assay as described earlier (Wee et al., 2002). Briefly, the MABTECH kit (cat. no. 3420M-2A) was used according to manufacturer’s instructions. PBMCs isolated on Lymphoprep were incubated for 24 h at 37 °C, 5 % CO₂, with the peptide YAPPIGQGI, corresponding to the defined Mamu-A*01-restricted CTL epitope. The released IFN-γ was captured on the bottom of the culture wells and visualized by combination of a second enzyme-coupled monoclonal antibody and a chromogenic substrate. The spots were counted using the AID ELISPOT Reader System (Autoimmun Diagnostika).

**RESULTS**

We have shown previously that codon optimization of HIV-1 envelope genes increased the level of expression and immunogenicity (Corbet et al., 2000; Vinner et al., 1999). Gene constructs were synthesized that encode the wild-type HIV-1Bx08 amino acid sequence but exclusively reflect the codon-usage of highly expressed human genes. The synthetic gp150Bx08 gene (syn.gp150Bx08) contains a stop codon after the transmembrane region to eliminate the putative internalization signal described in the cytoplasmic tail of the envelope glycoprotein for HIV-1 and SIV (Berlioz et al., 1999; LaBranche et al., 1995). The synthetic gp140Bx08 (syn.gp140Bx08) encodes gp120 plus the extracellular part of the gp41 that is largely responsible for oligomerization and which may contain important additional neutralizing epitopes similar to the 2F5 epitope (Chan et al., 1997; Muster et al., 1993). Thus, both the membrane-bound and the secreted constructs are designed for maximum expression of relevant primary HIV-1 envelope antigens.

**The neutralization sensitivity of HIV-1Bx08 resembles that of other primary prototype isolates**

The sensitivity to neutralization of the prototype primary HIV-1 isolates Bx08, JR-FL, Ba-L and SF162 was characterized using sCD4 and monoclonal antibodies (2G12, IgG1b12 and 2F5). Of the four primary virus prototypes examined, the Bx08 and JR-FL isolates are harder to neutralize by sCD4 and the monoclonal antibody IgG1b12 (Table 1). Thus, 5 to 50 times more sCD4 is required to obtain 80 % inhibition of Bx08 and JR-FL virus growth than is the case with the Ba-L and SF162 isolates. Inhibition by the 2G12 and IgG1b12 neutralizing antibodies is generally isolate-dependent. Whereas 12 and 5 μg ml^{-1} of IgG1b12 is required to neutralize HIV-1Bx08 and HIV-1Ba-L, respectively, one order of magnitude lower concentration of antibody is sufficient to neutralize HIV-1JR-FL and HIV-1SF162 (Table 1). Similarly, a higher 2G12 concentration is needed to neutralize HIV-1Bx08 compared to HIV-1SF162 and HIV-1Ba-L. Almost equal amounts of the somewhat less potent monoclonal antibody 2F5 are required to neutralize all four prototype isolates.

In addition, the neutralization sensitivity of the prototype primary isolates was examined using a panel of US patient sera with various potencies (Bures et al., 2000, 2002). Although some variations are seen, the titre measured against HIV-1Bx08 and HIV-1JR-FL were generally lower than for the HIV-1Ba-L and HIV-1SF162 isolates. Further neutralization studies of Bx08 were done with a panel of six sera from Danish patients. Results were essentially

http://vir.sgmjournals.org
similar to those obtained with the US sera, with IC_{80} values ranging from 7 to 199 with a median of 41 (data not shown). Thus, the neutralization sensitivity of the HIV-1 Bx08 isolate resembles that of a prototype primary isolate.

**Induction of antibody response in mice and guinea pigs after DNA immunization with codon-optimized envelope plasmid constructs**

Initially, we compared the antibody response upon immunization using either humanized env_{Bx08} DNA or commercially available recombinant gp120 protein. For this purpose BALB/c mice were immunized i.m. with plasmid DNA encoding synthetic gp120_{Bx08} (syn.gp120_{Bx08}). For comparison, other mice received subcutaneous (s.c.) injections of various amounts of recombinant gp120 protein in the saponin adjuvant QuadriA (Kamstrup et al., 2000). After the second immunization, mice receiving syn.gp120_{Bx08} DNA show a very strong IgG antibody response (Fig. 1). Although the increase in IgG titres is slightly slower than after recombinant protein injection, after 12 weeks the DNA construct achieved IgG titres comparable to those of rgp120IIIb in adjuvant-immunized animals.

IgG antibody response was observed in the guinea pigs that received i.m. DNA injections at weeks 0, 9 and 24 (Fig. 2a). The antibody response appeared after one injection and increased gradually reaching titres between 10^3 and 10^5. For comparison, guinea pigs were gene-gun inoculated at weeks 0, 3, 6, 9 and 24. IgG antibody titres measured in

**Table 1. Specific neutralization sensitivity of primary HIV-1 prototype isolates**

<table>
<thead>
<tr>
<th>HIV-1 isolate</th>
<th>Bx08</th>
<th>JR-FL</th>
<th>Ba-L</th>
<th>SF162</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCD4</td>
<td>50*</td>
<td>28</td>
<td>0.92</td>
<td>4.5</td>
</tr>
<tr>
<td>2G12</td>
<td>1.2</td>
<td>&lt;2.0</td>
<td>0.13</td>
<td>&lt;0.07</td>
</tr>
<tr>
<td>IgG1b12</td>
<td>12</td>
<td>0.05</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>2F5</td>
<td>27.9</td>
<td>30</td>
<td>30</td>
<td>33</td>
</tr>
</tbody>
</table>

Patient sera

<table>
<thead>
<tr>
<th>Serum</th>
<th>JAF-01</th>
<th>NLS-02</th>
<th>LEH-03</th>
<th>SCE-06</th>
<th>PRH-07</th>
<th>PFZ-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titre</td>
<td>20\†</td>
<td>172</td>
<td>80</td>
<td>112</td>
<td>36</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Titre</td>
<td>28</td>
<td>200</td>
<td>230</td>
<td>40</td>
<td>38</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Titre</td>
<td>15</td>
<td>&gt;320</td>
<td>&gt;320</td>
<td>110</td>
<td>130</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Titre</td>
<td>111</td>
<td>&gt;320</td>
<td>&gt;320</td>
<td>203</td>
<td>&gt;320</td>
<td>7</td>
</tr>
</tbody>
</table>

Negative serum

<5

\*Neutralization capacity of neutralizing monoclonal antibodies and sCD4 is expressed as the concentration \((\mu g \text{ ml}^{-1})\) needed to inhibit p24 antigen production by 80%.

\†Neutralization capacities of the antisera are expressed as the interpolated reciprocal dilution capable of inhibiting p24 antigen production by 80%.

**Fig. 1.** IgG antibody response in mice to rgp120_{IIIb}. The median ELISA titres and standard deviations are shown of five mice injected (s.c.) with 2 \(\mu g\) of recombinant gp120_{IIIb} protein in 10 \(\mu g\) QuadriA adjuvant (○) or i.m. with 200 \(\mu g\) syn.gp120_{Bx08} plasmid DNA (□). Immunizations of the two groups are indicated with arrows.

**Fig. 2.** Mean IgG antibody response in two guinea pigs immunized with syn.gp140_{Bx08} plasmid (□, ■) or syn.gp150_{Bx08} plasmid (△, △). Animals were immunized i.m. (a) and i.d. (gene gun) (b). Arrows indicate plasmid inoculations.
ELISA against recombinant gp120<sub>IIIb</sub> are shown in Fig. 2(b). For both the syn gp140<sub>Bx08</sub> and the syn gp150<sub>Bx08</sub> constructs, a high and consistent antibody response is seen at week 3 after a single inoculation. The antibody response reaches maximum titres after 12 weeks. For both constructs the magnitude of the antibody response did not decline greatly in the period between the initial immunizations (week 9) and the booster immunization given at week 24, regardless of the route of immunization.

**Priming of specific antibody and T cell responses in rhesus macaques after DNA immunization with syn gp140<sub>Bx08</sub> and syn gp150<sub>Bx08</sub> plasmid constructs**

For priming macaques we used 5 mg doses of plasmid DNA delivered by i.m. needle injection. The DNA immunizations were well tolerated.

We examined the gp120<sub>IIIb</sub>-specific IgG antibody response in the rhesus macaques by ELISA after DNA immunization with the synthetic gene constructs syn gp140<sub>Bx08</sub> and syn gp150<sub>Bx08</sub> at weeks 0, 4, 8 and 24 (Fig. 3a, b). Three i.m. injections with plasmid DNA containing either syn gp150<sub>Bx08</sub> or syn gp140<sub>Bx08</sub> were required to induce a primary antibody response that in most animals peaked 10 weeks post-immunization. Although a gradual decline in the IgG titres is seen following the primary DNA immunizations, the antibody response in macaques receiving either of the plasmid constructs peaked within just 2 weeks of a single plasmid DNA boost given at week 24 (e.g. Fig. 3a). This indicates the presence of memory induced by the initial DNA immunizations.

ELISPOT assays were used to monitor the development of specific T lymphocyte responses in the immunized rhesus macaques against the defined Mamu-A*01 MHC-I-restricted epitope YAPPIGGQI in the circulating blood of the immunized macaques (Table 2). Priming with the synthetic DNA constructs induced a high frequency of IFN-γ-producing T lymphocytes. Whereas three of four animals showed a T cell response after the second plasmid DNA immunization (week 6) macaque Gege needed four immunizations before an Env-specific T cell precursor population could be detected (week 26). The peaks in IFN-γ ELISPOT response at week 26 coincided with the peaks in antibody responses following immunization. Thus, both of the syn gp140<sub>Bx08</sub> and syn gp150<sub>Bx08</sub> genes delivered as naked plasmid DNA vaccines without adjuvants were able to prime both antibody and specific T cell responses in mice, guinea pigs and rhesus macaques against the HIV-1 envelope protein.

**Boosting of plasmid DNA vaccines by recombinant adenovirus vector**

Whereas control animals immunized with recombinant adenovirus alone reach antibody titres of approximately 10⁴ the DNA-primed macaques Gaffa, Gilda, Gege and Gloria reached titres of more than one order of magnitude higher and peaked approximately 2 weeks earlier. In the animals primed with syn gp150<sub>Bx08</sub> induction of memory by plasmid DNA was investigated by allowing an extended period of 24 weeks between the plasmid DNA boost and the subsequent recombinant adenovirus boost (Fig. 3a). During this period the antibody response gradually declined, indicating that antigen production from the inoculated plasmids had stopped. However, the adenovirus boost at week 48 initiated a rapid 3 log increase in antibody titres in Gaffa and Gilda that peaked 2 weeks after immunization. Thus, antibody titres increased with each
immunization and reached maximum upon rAd5 boosting. The development in antibody titres was reflected also in the relative avidity of the antisera, which were measured at each of the anti-gp120 antibody peaks (Table 4). The relative avidity of the antisera increased over time in all animals and seemed to be somewhat higher in the animals receiving the gp150 prime-boost regime. No further increase in antibody avidity was observed following the rAd5-induced antibody titre peak.

In all immunized macaques the adenovirus boost was followed also by increased specific T cell responses within 2 weeks (Table 2). The magnitude of the response ranged from 10 to 95 times, resulting in high frequencies of specific T cells (0–35–0–44 %) in the peripheral blood in three of four animals. Only Gaffa responded with a more moderate T cell frequency. Although the relatively high dose of recombinant adenovirus was able to generate both antibody and specific T cell responses without DNA priming higher antibody titres and specific T cell frequencies were achieved in the DNA-primed animals (Fig. 3c and Table 2).

### Table 2. Frequency of IFN-γ-releasing PBMCs upon specific stimulation with Mamu-A*01 epitope

<table>
<thead>
<tr>
<th>Animal</th>
<th>DNA priming</th>
<th>Weeks post-immunization</th>
<th>Weeks post-rAd5 boost</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0* 2 4* 6 8* 10 12 26</td>
<td>0† 2 4 8 10 12</td>
</tr>
<tr>
<td>Gaffa</td>
<td>syn.gp150Bx08</td>
<td>0‡ 0 0 200 0 245 90 40</td>
<td>34 414 135 95 23 0</td>
</tr>
<tr>
<td>Gilda</td>
<td>syn.gp150Bx08</td>
<td>0 0 330 1248 310 1490 790 1207</td>
<td>41 3888 168 100 35 0</td>
</tr>
<tr>
<td>Gege</td>
<td>syn.gp140Bx08</td>
<td>ND ND ND ND 0 0 0 50</td>
<td>438 4378 1040 18 ND 0</td>
</tr>
<tr>
<td>Gloria</td>
<td>syn.gp140Bx08</td>
<td>0 0 25 135 0 250 72 742</td>
<td>120 3462 188 65 130 0</td>
</tr>
<tr>
<td>Jez</td>
<td>–</td>
<td>– – – – – – –</td>
<td>0 362 215 25 85 0</td>
</tr>
<tr>
<td>Jolly</td>
<td>–</td>
<td>– – – – – – –</td>
<td>0 80 58 78 93 0</td>
</tr>
</tbody>
</table>

*Time of DNA immunization.
†Animals primed with syn.gp150Bx08 and syn.gp140Bx08 were boosted with rAd5 at weeks 48 and 32 respectively.
‡Values are frequency of IFN-γ releasing PBMCs (expressed as no. of spot-forming units per 10⁶ PBMCs) stimulated with the defined Mamu-A*01 epitope YAPPICGGQI.
ND, Not determined.

### Table 3. Neutralization of HIV-1Bx08 by macaque antiserum

<table>
<thead>
<tr>
<th>Animal</th>
<th>DNA priming</th>
<th>Weeks post-rAd5 boost*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≤0 2 4 8 10</td>
</tr>
<tr>
<td>Gaffa</td>
<td>Syn.gp150Bx08</td>
<td>&lt;5† &lt;5 7 57 &lt;5</td>
</tr>
<tr>
<td>Gilda</td>
<td>Syn.gp150Bx08</td>
<td>&lt;5 &lt;5 15 &lt;5 &lt;5</td>
</tr>
<tr>
<td>Gloria</td>
<td>Syn.gp140Bx08</td>
<td>&lt;5 &lt;5 &lt;5 &lt;5 &lt;5</td>
</tr>
<tr>
<td>Gege</td>
<td>Syn.gp140Bx08</td>
<td>&lt;5 &lt;5 &lt;5 &lt;5 7</td>
</tr>
<tr>
<td>Jez</td>
<td>–</td>
<td>&lt;5 &lt;5 21 &lt;5 5</td>
</tr>
<tr>
<td>Jolly</td>
<td>–</td>
<td>&lt;5 21 &lt;5 &lt;5 &lt;5</td>
</tr>
</tbody>
</table>

*Macques primed with syn.gp150Bx08 and syn.gp140Bx08 DNA (weeks 0, 4, 8 and 24) were boosted with rAd5 at weeks 48 and 32 respectively (see Fig. 3).
†Neutralization capacities of the antisera are expressed as the interpolated reciprocal dilution capable of inhibiting p24 antigen production by 80 %.

### Table 4. Relative avidity of antisera from DNA-primed macaques

<table>
<thead>
<tr>
<th>Animal</th>
<th>DNA priming</th>
<th>RNA immunization</th>
<th>rAd5 boost*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 26 2</td>
<td></td>
</tr>
<tr>
<td>Gaffa</td>
<td>syn.gp150Bx08</td>
<td>0·60† 0·68 0·75</td>
<td></td>
</tr>
<tr>
<td>Gilda</td>
<td>syn.gp150Bx08</td>
<td>0·54 0·63 0·79</td>
<td></td>
</tr>
<tr>
<td>Gloria</td>
<td>syn.gp140Bx08</td>
<td>0·57 0·64 0·70</td>
<td></td>
</tr>
<tr>
<td>Gege</td>
<td>syn.gp140Bx08</td>
<td>0·45 0·56 0·61</td>
<td></td>
</tr>
</tbody>
</table>

*Animals primed with syn.gp150Bx08 and syn.gp140Bx08 were boosted with rAd5 at weeks 48 and 32 respectively.
†The results are expressed as the normalized absorbance index at 4 M relative to the absorbance obtained without urea.
transient and appeared later than peaking antibody titres measured in ELISA (Fig. 3). Of the syn.gp140Bx08 DNA-primed animals, one sample showed measurable neutralization of primary HIV-1_Bx08 in repeated assays (Table 3, Gege). The animals receiving rAd5 alone also showed transient neutralizing activity that reach maximum activity as early as 2 or 4 weeks after immunization. Finally, no sera were able to provide significant cross-neutralization of the heterologous isolates HIV-1_R_F1, HIV-1_SF162, HIV-1_Ba-L or SHIV89-6P (data not shown).

**DISCUSSION**

We have described previously the construction of synthetic envelope genes derived from the early primary HIV-1_Bx08 isolate for use in vaccination experiments (Corbet et al., 2000). Here, we have found that the sensitivity of HIV-1_Bx08 to virus neutralization is comparable with three other isolates described as relatively neutralization resistant or prototypes of primary isolates (Gartner et al., 1986; Moore et al., 1995; Stamatatos & Cheng-Mayer, 1998). To obtain neutralization of HIV-1_Bx08, a high concentration of sCD4 was required. Similarly, in other studies including clinical isolates from different genetic subtypes and geographical origins it has been shown that concentrations of sCD4 lower than 50 μg ml⁻¹ only occasionally provide 80 % neutralization (Bures et al., 2000, 2002). In comparison with other patient isolates that typically require relatively high concentrations of the broadly neutralizing monoclonal antibodies 2G12 and IgG1b12 for significant neutralization, our findings show that the Bx08 isolate is somewhat more sensitive (Bures et al., 2000, 2002; Burton et al., 1994; Trkola et al., 1996). However, strain-dependant variation in neutralization sensitivity to monoclonal antibodies is often seen (Bures et al., 2000, 2002). From the panel of US patient sera used to further characterize the prototype primary isolates, a lower potency has been demonstrated against clade B and C primary isolates from different geographical origins (Bures et al., 2000, 2002).

The R5-tropic Bx08 virus was been isolated from a patient 8 months after seroconversion (Moog et al., 1997b). Neutralization studies using heterologous patient sera indicate the presence of common neutralizing antibody epitopes on the Bx08 envelope spikes (Moog et al., 1997a). In addition, in immunotypical analysis using monoclonal antibodies of a panel of viruses from different genetic subtypes and geographical origins, the Bx08 isolate was shown to contain antibody epitopes common to isolates from several other genetic clades (Nyambi et al., 2000). These properties in combination with the neutralization characteristics reported here and in other studies lead us to conclude that the primary HIV-1_Bx08 isolate is a relevant prototype vaccine strain.

Several studies have demonstrated much higher IgG titres upon immunization with recombinant envelope protein in comparison to titres upon immunizations with plasmid DNA wild-type envelope genes (Barnett et al., 1998; Gurunathan et al., 2000). For HIV envelope, results have probably been influenced by the Rev-dependent expression of the env gene and/or the suboptimal function of Rev in non-human cells (Trono & Baltimore, 1990). We have demonstrated previously that syn.gp120Bx08, encoding secreted gp120 Bx08, is Rev-independent and immunogenic in mice (Corbet et al., 2000), a circumstance that allows comparison of the immunogenicity of our vaccine construct with one of the few commercially available fully glycosylated recombinant envelope proteins. We found that mice immunized with plasmid DNA encoding humanized syn.gp120Bx08 show maximum titres comparable with mice injected with rgp120IIIb (Fig. 1). Doses of 10 μg per immunization of rgp120IIIb and the use of Freund’s adjuvants instead of QuadriA did not improve the antibody response against rgp120IIIb (data not shown) (Kamstrup et al., 2000; Sorensen, 2001). Moreover, the detection of specific IgG was performed in ELISA against recombinant gp120 protein of the IIIb strain homologous to the injected rgp120IIIb, which may bias the results in favour of the protein-immunized mice. Altogether, these results demonstrate the potential of codon-optimized envelope gene constructs and encouraged us to expand the immunization trials.

The antibody responses seen in guinea pigs are consistent with findings in earlier mouse studies. Persistent specific IgG responses are seen in both species after only one immunization with syn.gp140Bx08 and syn.gp150Bx08, either when injected i.m. or delivered to the skin by gene gun (Corbet et al., 2000; Vinner et al., 1999). However, transient envelope antibody titres are commonly observed in DNA-vaccinated larger animals, such as rabbits and macaques (Lu et al., 1996, 1998; Richmond et al., 1998; Robinson et al., 1999). Thus, the lower and transient increase of antibody titres reported in macaques after i.m. DNA inoculations are in contrast to antibody responses observed in guinea pigs and mice (Figs 2 and 3) (Corbet et al., 2000). In macaques, three injections of DNA are necessary before the primary rise in antibody is seen at weeks 4–8. The DNA boost at week 24 induced a faster antibody response, thus indicating the induction of memory by the DNA priming. Finally, other studies have included adjuvant with the DNA inoculations, which may possibly improve the DNA priming further (Shiver et al., 2002).

An rAd5 expressing the codon-optimized gp120Bx08 was administered i.m. to boost antibodies to epitopes primed by both syn.gp140Bx08 and syn.gp150Bx08 DNA. Boosting with rAd5 generates about 100-fold higher antibody titres than boosting with DNA. Antigen-specific antibody response has been demonstrated following recombinant adenovirus inoculations of macaques and other species (Bruce et al., 1999; Natuk et al., 1992; Prevec et al., 1991). Our results expand the use of recombinant adenovirus to include boosting after DNA priming of humoral immunity in addition to cellular immunity. Boosting of the syn.gp150Bx08
prime with rAd5 after an extended resting period of 24 weeks seemed to induce a greater increase in antibody titres and avidity. Whether the greater boost is related to the gp150Bx08 antigen or the extended resting period is unclear. However, for a variety of antigens and immunization methods the spacing between immunizations has been reported to affect the antibody response (Fuller et al., 1997; McCluskie et al., 1999).

Priming with the synthetic DNA induced a high frequency of T lymphocytes, comparable to that obtained after a DNA-prime MVA-boost regime or co-inoculation of DNA and IL-2 (Barouch et al., 2000; Wee et al., 2002). Only one macaque (Gege) showed no detectable T lymphocyte response before the DNA boost. In an earlier study, the reaction of this particular animal was slightly lower upon stimulation with superantigen, which could indicate that the magnitude of CD8+ responses in this animal is generally lower (Wee et al., 2002). Although results are not entirely comparable, a high frequency of specific T lymphocytes has been reported using a codon-optimized DNA vaccine encoding an SIV gag gene (Egan et al., 2000; Shiver et al., 2002). In addition, boosting of DNA-primed immune responses with other live recombinant viral vectors has earlier been shown to improve T cell responses (Allen et al., 2000; Hanke et al., 1999; Kent et al., 1998; Robinson et al., 1999). In our study, specific T lymphocyte responses were also improved using adenovirus expressing HIV-1 env genes. Furthermore, we demonstrate that the rAd5 boost (>10¹¹ p.f.u.) also induces a rapid increase in specific IgG antibody titres. Together, this confirms the induction of humoral and cellular memory by the DNA priming and suggests the use of adenovirus vectors for boosting of antibody responses to HIV-1 envelope proteins.

Guinea pigs failed to show neutralization. Since the guinea pigs were immunized with DNA only, it is possible that a boost using a viral vector is needed to improve neutralizing antibody titres against primary isolates such as HIV-1_Bx08. In contrast, using DNA-prime and rAd5-boosting, we have demonstrated neutralization of homologous virus by antisera from macaques. The neutralization activities of the antisera obtained from macaques upon rAd5 boosting are transient. Transient neutralizing antibody titres have been reported earlier after DNA immunization of macaques (Lu et al., 1996). Although neutralizing antibody titres against the Bx08 virus are moderate and generally lower than titres achieved with patient antisera, our results confirm that antibody-mediated neutralization of this primary isolate can be achieved by vaccination. Moreover, patient antibody responses have been continuously stimulated by their systemic infections for several years. In addition, whereas neutralization of homologous HIV-1 isolates is sometimes reported neutralization of heterologous primary isolates is rarely seen with sera from vaccinated animals.

A previous study has investigated inoculation of rhesus macaques with vaccines derived from the HIV-1_Bx08 isolate. In this study, strain-specific neutralizing antibodies were induced by different vaccination regimes that included Env, Gag and Pro (protease) from Bx08 delivered as virus-like particles and canarypox vectors (Montefiori et al., 2001). In the present experiment the in vitro neutralization is higher in antisera obtained from the macaques primed with syn.gp150 DNA than from macaques primed with syn.gp140 (Table 3). The neutralizing antibody titres did not correlate with ELISA antibody titres or relative avidity to rgp120Bx08. Thus, the differences measured in the macaques receiving the different immunization regimes seem mainly related to the antigen used for priming. The results emphasizes the importance of optimizing both antigen and immunization schedules. Whether qualitative differences can be detected in the pattern of antibody epitopes to which the neutralizing antisera react is being tested.

In conclusion, the DNA prime/rAd5 boost is an effective regime for induction of both humoral and cellular immunity in macaques. The codon-optimized DNA vaccines with primary HIV-1 envelope genes are immunogenic in macaques, and induce memory, high and specific antibody and cellular immune responses. The replication-deficient recombinant adenovirus vector could significantly boost the responses to obtain neutralizing antibodies against HIV-1_Bx08. The results presented here are encouraging for further immunization studies using humanized syn. gp150Bx08 envelope constructs in combination with other optimized primary envelope antigens to broaden the neutralizing antibody response in prime-boost regimes including a viral vector.

ACKNOWLEDGEMENTS

We would like to thank Dr David C. Montefiori for his supervision and the generous supply of materials when setting up the neutralization assay and for performing the SHIV96K neutralization assay. We acknowledge Nanna Skall Sorensen and Claus Kock for the protein immunization data in mice. We acknowledge the assistance of the technicians Vivi Brummer, Janne Brast, Nina Frelund, Irene Jensen, Birgit Knudsen and Anne Lyhning. This work was supported by grants from the Danish Research Council’s Technology by Highly Oriented Research (THOR) Programme, the Velux Foundation, the Danish AIDS Foundation, and from grants by NIAID (H. Ertl).

REFERENCES


Moore, J. P., Cao, Y., Qing, L., Sattentau, Q. J. & 7 other authors (1995). Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. J Virol 69, 101–109.


