We have identified an acceptor site on HIV gp120, where foreign protein sequences can be inserted while retaining the native conformation of gp120. The resulting hybrids showed dual antigenicity, normal glycosylation, and high affinity binding of the CD4 receptor. This site allows insertion of highly immunogenic proteins such as core antigen of hepatitis B virus. By combining the immunogenicity of the carrier protein with the antigenicity of gp120, these hybrids may lead to modified HIV-1 antigens with enhanced immunogenicity.

Key Words: HIV vaccine; immunogenicity; gp120; particle assembly.

Materials and Methods

Plasmid construction. DNA coding for Hepatitis B core antigen (amino acids 1–149) was PCR amplified from pECO63 (ATCC, Manassas, VA) using primers CGGAATTCATGGACATTGACC (JP1) and CGGGGTACCACAGTAGTTTCCGG (JP2). The 450 bp DNA product was cut with EcoRI and KpnI and ligated into plasmid pSM-WT (HXB2) (19) at the internal KpnI site corresponding to amino acid 42 of envelope gp120. The resulting plasmid was cut with EcoRI and HindIII, ending at amino acid 639, and ligated into pGEM (Promega, Madison, WI). The resulting plasmid pGEM/CE codes for residues 1 to 149 of Hepatitis B core antigen in tandem with HIVIIIb envelope glycoprotein residues 42 to 639.

A modified precore leader sequence was amplified from pECO63 DNA with primers CGGCGTCGACAGGAGGCTGTAGGC (AL9) and CCGGAATTCGGAAGCTTGAACAGT (AL10). The 150 bp DNA product was ligated into pGEM/CE, producing plasmid pGEM/pcl/CE with a shortened precore leader followed by core/env sequences. The modified leader sequence coded for amino acids 1–20 of the natural precore leader, but lacked residues 21–29, which interfere with self-assembly of core antigen particles (20, 21). Alternatively, the leader sequence coding for amino acids 1–26 of influenza hemagglutinin was used, because it is permissive of assembly (22). This sequence was amplified from influenza virus cDNA (a gift of Dr. Roland LeVanderowski, CBER) with primers CCGTCGACAGCAAAAGCAGGGG and CCGAATTCGCTGTTGTGATTTCC and ligated to the gene for core antigen.

Plasmid pSC65 was obtained from Dr. B. Moss, NIH (23) and modified by adding a new SpeI site to the multiple cloning site. Plasmid pSC65 was digested with Sall and SpeI, and ligated
into modified pSC65, creating plasmid pJK2 (Fig. 1). Recombinant vaccinia virus containing J K2 was made by coinfection and transfection of CV-1 cells. Cell lysates and culture supernatants were screened for core/env expression by capture ELISA in wells coated with monoclonal anti-gp120, followed by detection with polyclonal human antibodies to gp120. The other hybrids were made by substituting different forms of gp120 for the env sequence of J K2. Vaccinia recombinant PE50 was a gift of Dr. Pat Earl, NIH.

Hybrid expression. CV-1 cells and tk− cells were obtained from Dr. Michael Merchlinsky (CBER, Bethesda, MD). CV-1 monolayers were infected with recombinant vaccinia virus at moi 3:1 and incubated for 2 to 3 days at 37°C. Antigen expression was assayed by capture ELISA.

Western blot. Following electrophoresis on a 4-12% polyacrylamide gel and transfer to nitrocellulose, bands were developed with polyclonal goat anti-gp120 env 2–3 (1:5000) and peroxidase conjugated anti-goat immunoglobulin (24). In preliminary experiments, western blot demonstrated the size and CD4 binding of core/env hybrid transiently expressed in COS cells and immunoprecipitated with CD4-Ig.

CD4 binding assay. As described previously (25), 0.1 to 10 nM concentrations of core/envelope hybrid were mixed with excess CD4-Ig (20 nM) for one hour at room temperature to allow complex formation. Bound complexes were captured in ELISA wells coated with monoclonal anti-gp120 (No. 13-105-100, Advanced Biotech., Columbia, MD) and detected with alkaline phosphatase conjugated goat anti-human IgG (Cappel Labs, Durham, NC), which binds the Fc tail of CD4-Ig.

Immunization. Mice received three doses of core/env hybrid (1 μg/dose) intraperitoneally, at 0, 5, and 9 weeks, as described previously (26). Antibodies were measured by ELISA on soft plastic plates (Falcon MicroTest III, 3912, Becton Dickinson, Oxnard, CA) coated with gp120 (Protein Sciences Corp., Meriden, CT) or hepatitis B core antigen (Biogen, Inc., Cambridge, MA). Antibody titer was defined as the dilution giving 1/4 of the plateau value of OD410.

RESULTS

Expression of core/env hybrids. A series of core antigen/gp120 hybrids were generated by linking core antigen residues 1–149 to large fragments of HIV envelope (Fig. 1). Hybrid J K2 consisted of full length gp120 plus nearly the entire ectodomain of gp41. In hybrid MR2, this was shortened to gp120 alone, while MR9 was further shortened by deleting the V1V2 variable region, as described by Pollard (16). An endoplasmic reticulum retention signal, KDEL was added to hybrid MR14, to increase its intracellular concentration to the threshold for assembly (15, 27). When core antigen (MR3) or gp120 controls (PE50) were expressed in the same way, they formed native proteins which assembled normally or bound CD4 with high affinity.

Each hybrid was expressed by recombinant vaccinia virus under control of a synthetic early and late promoter capable of high-level protein expression (28). Hybrid proteins were detected in cell lysates and culture supernatants by antigen capture ELISA, or by Western blot. JK2 ran as a single band of about 140KD (Fig. 2, lane 2). Dual antigenicity of JK2 was demonstrated by capturing the hybrid on plates coated with a monoclonal antibody to gp120, followed by detection with polyclonal antibodies to core antigen (not shown).

CD4 binding. Core/env hybrids J K2, MR2, and MR9 were incubated with CD4-Ig in solution, and bound complexes were captured in ELISA wells coated with monoclonal anti-gp120 (Fig. 3). J K2, MR2, and MR9 hybrids bound CD4-Ig with high affinity (kDa of 1 to 2 nM), indistinguishable from native gp120 expressed by vaccinia (PE50). Since CD4 binding depends on the native conformation, the results suggest that the CD4 binding site folds correctly in both hybrids, despite insertion of core antigen. Val 42 functions as an insert acceptor site on gp120, where foreign
sequences can be inserted without disrupting the native folding.

Affinity purification. Core-envelope hybrid was adsorbed on Lentil lectin-Sepharose beads, washed, and eluted with 0.5 M alpha-methyl mannoside (Fig. 4), as described (28). Most proteins passed through the column, while the core-envelope hybrid stuck to the column and was eluted by alpha-methyl mannoside. The crude lysate contained 1.4 mg/ml protein and 0.66 μg/ml antigen. The peak eluate fraction contained 0.03 mg/ml protein and 2.6 μg/ml antigen, for a purification of more than 150-fold and a recovery of greater than 50%. Affinity purification on lentil lectin, plus its size on Western blot, indicate that core/env hybrid was fully glycosylated.

Immunogenicity in mice. Previous studies have shown that the murine immune response to native gp120 varied by more than 100-fold, depending on MHC type (26). H-2a mice (B10.A) were high responders, while H-2s (B10.S) and H-2b (B6) were low responders. These MHC linked differences indicate the critical role of T cell help in generating an immune response to gp120. We immunized the same strains of mice with JK2 core/env hybrid, to determine whether T helper effects from core antigen could enhance the response to gp120. Mouse antibodies were titered on ELISA plates coated with recombinant gp120 (Fig. 5, upper panel) or core antigen (lower panel), to determine the response to each component separately.

Following two doses of core/env hybrid JK2, B10.A mice made high-titered antibodies to both core antigen and gp120 (Fig. 5, upper panel), just as they had to gp120 alone (26). In contrast, B6 mice responded weakly to gp120 in the hybrid, with antibody titers no greater than those elicited by gp120 alone, and B10.S mice responded as weakly to core/env hybrid as they did to gp120 alone (26). Despite the strong response of control B10.S mice to assembled core antigen (Fig. 5, lower panel), they responded weakly to core antigen in the hybrid. In genetic high responders, the hybrid elicited a vigorous response to both antigens. In low responder strains, however, the weak response to gp120 was accompanied by a weak response to core antigen. In order to enhance the immune response to gp120, carrier/gp120 hybrids may need to elicit a much stronger helper T cell response to the carrier protein itself.

DISCUSSION

We have identified an insertion site at amino acid 42 of gp120, where foreign protein sequences as large as core antigen can be linked to gp120 without disturbing the native conformation. Dual antigenicity was demonstrated by antigen capture ELISA and by immunizing mice with the hybrid and eliciting antibodies to both core antigen and gp120 in high responders. Normal glycosylation was shown by size on western blot and affinity chromatography on lentil lectin columns. Native folding was shown by high affinity binding of gp120 to monoclonal anti-gp120.
CD4. However, the core/env hybrids did not assemble particles, even though native core antigen, expressed in the same way, did assemble. Lacking assembly, the hybrids did not enhance the immune response to gp120, and even core antigen in the hybrid was much less immunogenic than in native core antigen particles.

Our approach was based on the observation that multimeric vaccine antigens, such as hepatitis B surface antigen (HBsAg) (29) and L1 capsid protein of human papilloma virus (30), are up to 1000-fold more potent as particles than the same weight of protein monomers. Each hybrid was designed to address an issue in particle assembly. MR2 and MR9 employed progressively shorter envelope sequences, in order to reduce steric hindrance of core antigen assembly. We added an endoplasmic reticulum retention signal to MR14, so it could reach the threshold needed for assembly. The natural precore leader sequence prevents assembly, so it was truncated in JK2 and substituted with a permissive leader sequence from influenza hemagglutinin (22) for all subsequent constructs.

Particle assembly may depend on compatibilities other than just size. For example, carrier proteins that form flexible pleomorphic particles may incorporate gp120 more easily than core antigen, which forms rigid icosahedral particles. Hydrophobic proteins that localize to a lipid/water interface may duplicate the natural milieu of gp120 on the viral surface. Proteins that follow the same post-translational processing pathways as gp120 may be more suitable than core antigen, which assembles, unglycosylated, in the cytoplasm. In summary, these hybrids should provide a useful starting point for linking gp120 to a variety of carrier proteins capable of enhancing immunogenicity, while retaining the native structure needed to elicit broadly crossreactive neutralizing antibodies against HIV virus.

REFERENCES


