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Enhancement of Antibodies to the Human Immunodeficiency Virus Type 1 Envelope by Using the Molecular Adjuvant C3d

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DNA vaccines expressing the envelope (Env) protein of the human immunodeficiency virus have been relatively ineffective at generating high-titer, long-lasting, neutralizing antibodies in a variety of animal models. In this study, the murine and human homologues of the complement component, C3d, were used in a DNA vaccine to enhance the titers of antibody to Env. Initially, plasmids expressing a secreted form of Env (sgp120) fused to one, two, or three copies of the murine homologue of C3d (mC3d) were constructed. Mice were inoculated with four vaccinations of DNA or two DNA vaccinations, followed by two boosts of affinity-purified gp120 protein. Analyses of titers demonstrated that multiple copies of mC3d coupled to sgp120 induced long-lasting, high-titer anti-Env antibody. Priming mice with sgp120-mC3d-DNA, followed by inoculation of purified gp120 protein, elicited the strongest antibody titers; however, the avidity maturation of the antibody was accelerated in the mice inoculated with sgp120-mC3d$_3$-DNA. In addition, DNAs expressing sgp120 fused to three copies of the human homologue of C3d (hC3d$_3$) efficiently enhanced the anti-Env antibody in rabbits. Lastly, antisera from both mice and rabbits vaccinated with DNA expressing sgp120-C3d$_3$ elicited higher titers of neutralizing antibody than did nonfused forms of Env. These results indicate that C3d, conjugated to sgp120, enhances the antibody responses to Env compared to non-C3d fused forms of Env, and this approach may be one way to overcome the poor ability of DNA vaccines to generate antibodies to Env.

DNA vaccination (genetic vaccination) induces protective immunity against a variety of pathogens (for reviews, see references 20, 32, 53, and 54). These genetic vaccines consist of eukaryotic expression plasmids that are inoculated into target cells and translated into proteins (20). Previous studies have demonstrated that DNA vaccination effectively induces both humoral and cellular immune responses to immunogens from diverse infectious agents (20, 32, 53, 54). However, DNA immunizations have been less successful at generating neutralizing antibodies against human immunodeficiency virus type 1 (HIV-1) (54). Unlike most immunogens, multiple DNA immunizations are required to elicit even modest titers of neutralizing antibody to the HIV envelope (Env) glycoprotein (4, 5, 11, 18, 33, 34, 43, 59). In addition, the antibody responses raised by DNA vaccination, like those to Env (gp120) subunit immunizations, are transient, rising and falling with each successive immunization (26, 42, 51). In HIV-infected patients or in experimentally SIV-infected rhesus macaques, specific antibodies require 6 to 8 months to achieve affinity maturation and may be associated with the appearance of neutralizing antibody (17). Therefore, we sought to increase the efficacy of DNA vaccines expressing HIV Env by using a component of the innate immune system, C3d, in order to enhance antibody titer, the affinity maturation, and the virus-neutralizing ability of the elicited antibody.

In the human immune system, C3d is one of the final degradation products of the third complement protein, C3. The C3d receptor, CD21 or CR2, is located on B cells, follicular dendritic cells (FDC), and possibly some epithelial tissues (33, 34). One consequence of complement activation is the covalent attachment of the C3d to antigen. On B lymphocytes, C3d-CD21 interaction stimulates B cells amplifying lymphocyte activation (19). Recently, our laboratory and others have demonstrated that C3d can enhance antibody responses directed toward a specific antigen encoded by a DNA vaccine (26, 38, 55, 56, 62). A DNA vaccine expressing a fusion of hemagglutinin (HA) from influenza virus or measles virus fused to three copies of the murine homologue of C3d (mC3d) achieved an earlier and more efficient immune response (26, 38, 55). These results demonstrated that mice vaccinated with DNA expressing a secreted, soluble HA-mC3d$_3$ immunogen elicited antibody that underwent more rapid affinity maturation than antibody generated by non-C3d fused forms of secreted or transmembrane of HA. This resulted in more rapid appearance of hemagglutination inhibition activity, neutralizing titers, and protective immunity (26, 38, 55). In addition, our laboratory has used a similar approach with the HIV-1 envelope (gp120) fused at the carboxyl terminus with C3d$_3$ (56). By using DNA vaccination, BALB/c mice were inoculated and assayed for enhanced immune responses. The fusion constructs induced higher antibody responses to Env and a faster onset of affinity maturation than did the respective wild-type gp120 sequences (56).

The present study differs from our past studies in three significant aspects. (i) In order to determine the number of C3d genes necessary to elicit the maximum immune response by DNA vaccination, mice were vaccinated with DNA expressing sgp120 fused to one, two, or three copies of mC3d. (ii)

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Even though mice vaccinated with sgp120-mC3d$_3$-DNA elicited enhanced antibody responses, these same antibodies were unable to neutralize HIV infection. Therefore, in the present study, vaccination with DNA was followed by protein boosts of gp120 in order to enhance the neutralizing antibody titers to Env. (iii) The human homologue of C3d was fused to sgp120 and examined for anti-Env immunogenicity and neutralizing capability.

MATERIALS AND METHODS

Plasmid DNA. pTR600, a eukaryotic expression vector, has been described previously (27). Briefly, the vector was constructed to contain the cytomegalovirus immediate-early promoter plus intron A for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal for termination of transcription (Fig. 1). The vector contains the ColE1 origin of replication for prokaryotic replication and the kanamycin resistance gene for selection in antibiotic media.

HIV envelope sequences from the isolates, ADA, IIIB, JRFL, and 89.6 encoding almost the entire gp120 region (Fig. 1A) and C3d sequences were cloned into the pTR600 vaccine vector by using unique restriction endonuclease sites (Fig. 1B). The gp120 segment encodes a region from amino acids 32 to 465 and ended with the amino acid sequence VAPTRA. The first 32 amino acids were deleted from the N terminus of each sgp120 and replaced with a leader sequence from the tissue plasminogen activator (tpA). The vectors expressing sgp120-mC3d fusion proteins were generated by cloning one, two, or three tandem repeats of the mouse homologue of C3d in frame with the sgp120 expressing DNA. The construct design was based upon the method of Dempsey et al. (19). Linkers composed of two repeats of four glycines and a serine [$(G_4S)_2$] were fused at the junctures of Env and C3d and between each C3d repeat. Potential proteolytic cleavage sites between the junctions of Env and the junction of C3d$_3$ were mutated by ligating *Bam*HI and *Bgl*II restriction endonuclease sites to mutate an Arg codon to a Gly codon (6). Vectors expressing sgp120-hC3d$_3$ were generated by cloning three copies of the human homologue of C3d (generously donated by Adprotech, Saffron-Walden, United Kingdom) in frame with the sgp120 sequence. Each hC3d gene was optimized to reduce possible recombination of the tandem C3d genes.

The plasmids were amplified in *Escherichia coli* DH5α; purified by using endotoxin-free, anion-exchange resin columns (Qiagen, Valencia, Calif.); and stored at −20°C in distilled H_2O. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined based on the optical densities (ODs) at 260 and 280 nm, and therefore each DNA vaccine inoculation contained ≥50 fg/μg of DNA/inoculation.

Animals and DNA immunizations. Six- to eight-week-old BALB/c mice (Harlan Sprague-Dawley, Indianapolis, Ind.) were used for inoculations. Mice, housed with free access to food and water, were cared for under U.S. Department of Agriculture guidelines for laboratory animals. Mice were anesthetized with 0.03 to 0.04 ml of a mixture of 5 ml of ketamine HCl (100 mg/ml) and 1 ml of xylazine (20 mg/ml). Gene gun immunizations were performed on shaved abdominal skin by using the hand-held Bio-Rad gene delivery system as described previously (30, 46, 47). Mice were immunized with two gene gun doses containing 2 μg of DNA per 0.5 mg of approximately 1-μm gold beads (Bio-Rad, Hercules, Calif.) at a helium pressure setting of 400 lb/in^2. Six- to eight-week-old New Zealand White rabbits (Robinson Farms, Clemons, N.C.) were vaccinated with 250 μg of DNA in 0.9% saline by intramuscular injection (125 μg/leg). Purified gp120 (Advanced Biotechnologies, Inc., Columbia, Md.), emulsified in Freund complete adjuvant (1:1 in saline; Sigma, St. Louis, Mo.), was inoculated into the leg of each mouse (30 ng) and each rabbit (5 μg).

Transfections and expression analysis. The human embryonic kidney cell line, 293T, was transfected (at 5×10^5 cells/transfection) with 2 μg of DNA by using 12% Lipofectamine (Life Technologies, Grand Island, N.Y.) according to the manufacturer's guidelines. Supernatants were collected and stored at −20°C. Cell lysates were collected in 300 μl of radioimmunoprecipitation assay lysis buffer (0.05 M Tris-HCl, pH 8.0; 0.1% sodium dodecyl sulfate [SDS]; 1.0% Triton X-100; 2 mM phenylmethylsulfonyl fluoride; 0.15 M NaCl) and stored at −20°C. Quantitative antigen capture enzyme-linked immunosorbent assays (ELISAs) were conducted as previously described (56).

For Western hybridization analysis, 1.5% of supernatant was diluted 1:2 in SDS sample buffer (Bio-Rad) and loaded onto a 10% polyacrylamide–SDS gel. The resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad) and incubated with a 1:3,000 dilution of polyclonal human HIV-infected patient antisera in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% fetal calf serum. After an extensive washing, bound human antibodies were detected by using a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-human antiserum and enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom). In order to analyze the presences of anti-hC3d$_3$ antibodies in sera from vaccinated rabbits, 15 μl of sera was incubated for 45 min with 100 μl of hC3d$_3$ containing supernatant from 293T cells transfected with pTR600-tpA-hC3d$_3$. Anti-hC3d$_3$-depleted serum was used to detect hC3d$_3$ or sgp120 proteins by Western blot analysis.

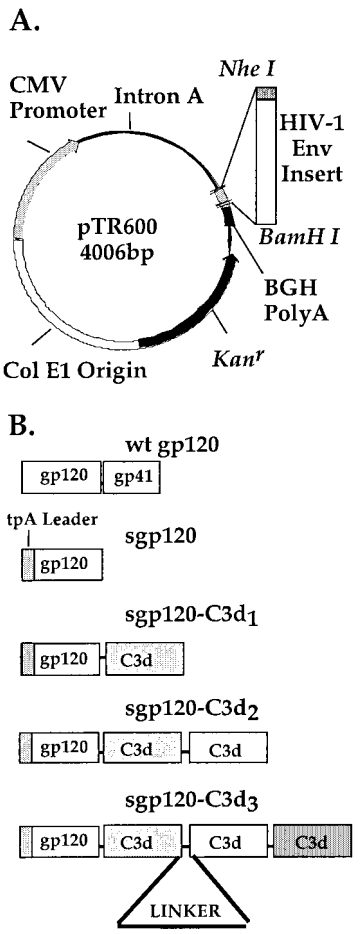

FIG. 1. Schematic representation of vector DNA vaccine constructs. (A) The pTR600 vector contains the cytomegalovirus immediate-early promoter plus intron A for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation terminator (BGH PolyA) for the termination of transcription. The vector also contains the ColE1 origin of replication for prokaryotic replication, as well as the kanamycin resistance (Kan^r) gene for selection in antibiotic media. Inserts were cloned into the vector by using the *Nhe*I and *Bam*HI restriction endonuclease sites directly 3′ to the tpA sequence. (B) The first schematic represents the wild-type, transmembrane form of the Env protein. The second schematic represents the secreted gp120 form of the Env. The third schematic represents the sgp120-C3d$_1$ construct used as a vaccine insert. The fourth schematic represents the sgp120-C3d$_2$ construct used as a vaccine insert. The fifth schematic represents the sgp120-C3d$_3$ construct used as a vaccine insert. Linkers composed of two repeats of four glycines and a serine [$(G_4S)_2$] were fused at the junctures of Env and C3d and between each C3d repeat.

Immunological assays. An endpoint ELISA was performed to assess the titers of anti-Env immunoglobulin G (IgG) in immune serum by using purified HIV-1 IIIB gp120 Chinese hamster ovary (CHO) cell-expressed protein (Quality Biologicals, Inc., Gaithersburg, Md.) to coat plates as described previously (51).

Mouse sera from vaccinated mice were allowed to bind and subsequently were detected by anti-mouse IgG conjugated to horseradish peroxidase. Endpoint titers were considered positive that were twofold higher than the background level. Sera that had background titers at the minimum dilution tested were recorded as less than 1:100 (<100).

Avidity ELISAs were performed similarly to serum antibody determination ELISAs up to the addition of samples and standards (17, 28, 35, 48, 50, 60). Samples were diluted to give similar concentrations of specific IgG as indicated by the OD. Plates were washed three times with 0.05% PBS-Tween 20. Different concentrations of the chaotropic agent, sodium thiocyanate (NaSCN) in PBS, were then added (0, 1, 1.5, 2, 2.5, and 3 M NaSCN). Plates were allowed to stand at room temperature for 15 min and then washed six times with PBS-Tween 20. Subsequent steps were performed similarly to the serum antibody determination ELISA. The percentage of initial IgG was calculated as a percentage of the initial OD. All assays were done in triplicate.

Neutralizing antibody assays. Antibody-mediated neutralization of HIV-1 IIIB and 89.6 was measured in an MT-2 cell-killing assay (40). Briefly, cell-free virus [50 μl containing 500 50% tissue culture infective dose(s) of virus] was added to multiple dilutions of serum samples in 100 μl of growth medium in triplicate wells of 96-well microtiter plates and incubated at 37°C for 1 h before MT-2 cells were added (5×10^4 cells in 100 μl added per well). Cell densities were reduced, and the medium was replaced after 3 days of incubation when necessary. Neutralization was measured by staining viable cells with Finter's neutral red when cytopathic effects in control wells were >70% but <100%. The percentage of protection was determined by calculating the difference in absorbance (A_{540}) between test wells (cells plus virus) and dividing this result by the difference in absorbance between cell control wells (cells only) and virus control wells (virus only). Neutralizing titers are expressed as the reciprocal of the plasma dilution required to protect 50% of cells from virus-induced killing. This 50% cutoff corresponds to a 90% reduction in viral antigen synthesis.

Statistics. For statistical analysis of the difference between sgp120 fused to multiple copies of murine C3d and sgp120 alone or fused to one copy of murine C3d, the Student *t* test was used. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Expression of plasmids. Vaccine plasmids, expressing a secreted form of Env (sgp120), were constructed by using the previously described pTR600 vector (56). The first set of vaccine plasmids expressed the sgp120 from the HIV-1 isolate IIIB ($sgp120_{IIIB}$) Env or fused to one ($sgp120_{IIIB}$-$mC3d_1$), two ($sgp120_{IIIB}$-$mC3d_2$), or three ($sgp120_{IIIB}$-$mC3d_3$) copies of murine C3d (Fig. 1A). The molecularly cloned gp120 region represented the entire surface domain of Env but excluded the oligomerization and transmembrane domains and the cytoplasmic regions (Fig. 1B). The $sgp120_{IIIB}$-mC3d fusion proteins were generated by cloning tandem repeats of the mC3d (19) in frame with the sgp120 gene (Fig. 1B). In addition to the $sgp120_{IIIB}$ vaccines, *env* genes were cloned from each of two, HIV isolates, ADA and 89.6, and these same *env* genes were fused to $mC3d_3$.

DNA vaccines efficiently expressed each secreted form of gp120 and gp120-$mC3d_3$ protein (Fig. 2). However, expression was lowered by two- to fourfold with plasmids expressing C3d fused forms of Env as determined by antigen capture ELISA (data not shown). Human 293T cells were transiently transfected with 2 μg of plasmid, and both supernatants and cell lysates were assayed by ELISA with HIV-Ig. As observed previously (56), ca. 90% of the Env protein was present in the supernatant for both sgp120 and sgp120-$C3d_3$-DNA transfected cells.

Western blot analyses revealed sgp120 and sgp120-C3d proteins of the expected sizes (Fig. 2). Using human patient polyclonal antisera (HIV-Ig), Western blot analysis showed the expected broad band of 115 to 120 kDa corresponding to gp120 (Fig. 2). A higher-molecular-weight band was seen for each of the sgp120-mC3d fusion proteins (Fig. 2). Consistent with the antigen capture assay, intense protein bands were present in the supernatants of cells transfected with sgp120-DNA. No evidence for the proteolytic cleavage of the sgp120-C3d fusion protein was seen by Western analysis.

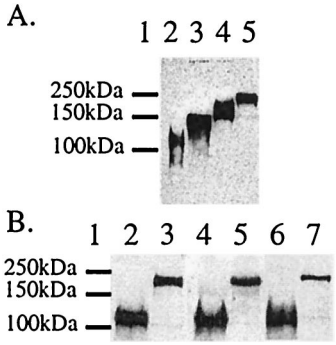

FIG. 2. Western hybridization to detect expression of vaccine constructs in vitro. Human embryonic kidney cells (293T) were transfected with 2 μg of each vaccine plasmid. Supernatant was collected, and 1.5% of total volume was subjected to electrophoresis on a 10% polyacrylamide gel. Each blot was probed with HIV-Ig followed by anti-human IgG conjugated to horseradish peroxidase. Proteins were detected by using a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-human antiserum and enhanced chemiluminescence. (A) Lane 1, molecular mass marker; lane 2, $sgp120_{IIIB}$-DNA; lane 3, $sgp120_{IIIB}$-$mC3d_1$-DNA; lane 4, $sgp120_{IIIB}$-$mC3d_2$-DNA; lane 5, $sgp120_{IIIB}$-$mC3d_3$-DNA. (B) Lane 1, molecular mass marker; lane 2, $sgp120_{IIIB}$-DNA; lane 3, $sgp120_{IIIB}$-$mC3d_3$-DNA; lane 4, $sgp120_{ADA}$-DNA; lane 5, $sgp120_{ADA}$-$mC3d_3$-DNA; lane 6, $sgp120_{89.6}$-DNA; lane 7, $sgp120_{89.6}$-$mC3d_3$-DNA.

Antibody response to Env gp120 fused with mC3d DNA immunizations. BALB/c mice were vaccinated by DNA-coated gold particles via gene gun with a 1-μg dose inoculum. Mice were vaccinated at day 1 and then boosted at week 6. DNA plasmids expressing sgp120 fused to multiple copies of mC3d ($sgp120_{IIIB}$-$C3d_2$ or $sgp120_{IIIB}$-$mC3d_3$) raised higher titers of antibody as determined by ELISA than the $sgp120_{IIIB}$-DNA or $sgp120_{IIIB}$-$mC3d_1$-DNA (Fig. 3A). In addition, these mice had detectable titers of anti-Env antibody after the second inoculation. In vaccinated mice, the temporal pattern for the appearance of anti-Env antibodies revealed titers that were boosted after each inoculation with DNA expressing $sgp120_{IIIB}$-$C3d_2$ or $sgp120_{IIIB}$-$mC3d_3$.

At week 12, mice were divided into two groups and vaccinated two additional times (weeks 12 and 18). Half of the mice were inoculated with the same DNA vaccine given at weeks 0 and 6, and the other half were inoculated with 10 μg of purified $gp120_{IIIB}$ protein (Quality Biologicals). Mice primed and boosted with $sgp120_{IIIB}$-$mC3d_3$-DNA had an average anti-Env endpoint titer that peaked at 1:7,000 at week 14. This titer was maintained for an additional 12 weeks (week 30) after the final inoculation. In contrast, mice primed with $sgp120_{IIIB}$-$mC3d_3$-DNA and boosted with $gp120_{IIIB}$ protein also had an average peak titer of 1:7,000; however, it took two protein boosts to achieve this same titer (Fig. 3B). In comparison, slightly higher titers (1:12,800) were observed in sera from an HIV-infected patent (HIV-Ig). Interestingly, mice vaccinated with four inoculations of $sgp120_{IIIB}$-DNA or $sgp120_{IIIB}$-$mC3d_1$-DNA had

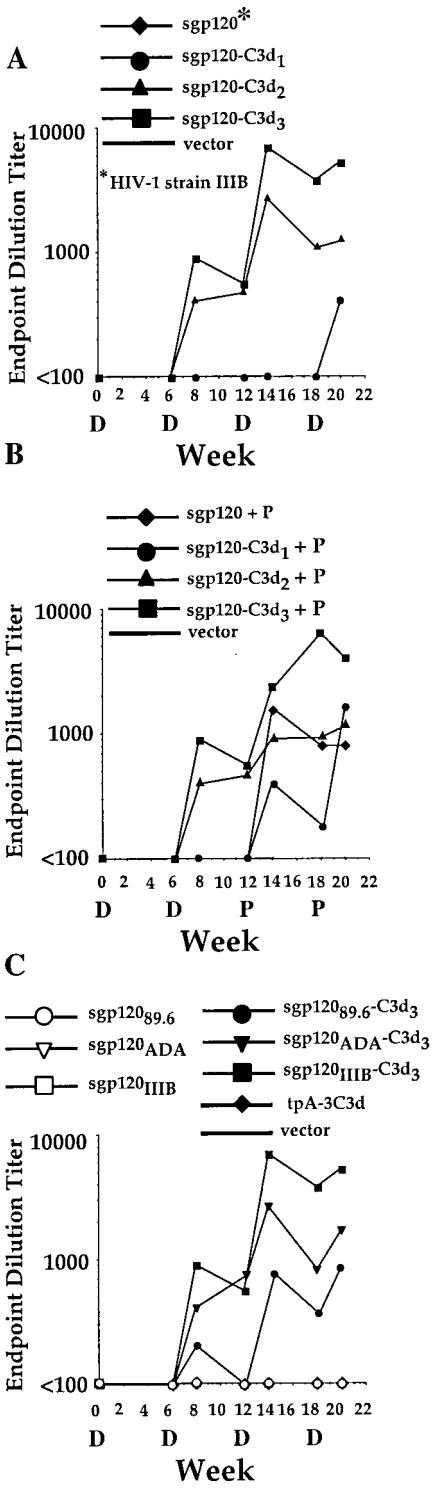

FIG. 3. Anti-Env IgG raised by DNAs expressing sgp120 proteins in gene gun-vaccinated mice. (A) Mice were primed with DNA at day 0 and boosted at weeks 6, 12, and 18. Sera were obtained from mice at weeks 0, 6, 8, 12, 14, 18, and 20. Symbols: —, vector; ◆, $sgp120_{IIIB}$-DNA; ●, $sgp120_{IIIB}$-$mC3d_1$-DNA; ▲, $sgp120_{IIIB}$-$mC3d_2$-DNA; ■, $sgp120_{IIIB}$-$mC3d_3$-DNA. (B) Mice were primed with DNA at day 0 and 6 and then were boosted with $sgp120_{IIIB}$ at weeks 12 and 18. (C) Mice were primed with DNA at day 0 and boosted at weeks 6, 12, and 18. Sera were obtained from mice at weeks 0, 6, 8, 12, 14, 18, and 20. Symbols: —, vector; ○, $sgp120_{89.6}$-DNA; ▽, $sgp120_{ADA}$-DNA; □, $sgp120_{IIIB}$-DNA; ●, $sgp120_{89.6}$-$mC3d_3$-DNA; ▼, $sgp120_{ADA}$-$mC3d_3$-DNA; ■, $sgp120_{IIIB}$-$mC3d_3$-DNA; tpA-$mC3d_3$. Sera collected at the indicated times from each mouse were assayed for specific IgG levels by ELISA. Then, 96-well plates were coated with recombinant gp120 protein-derived CHO cells expressing the HIV-1 isolate, IIIB. Data are averages for 5 to 10 mice. Preimmune sera from mice had no detectable specific IgG. Endpoint dilution titers were conducted by diluting the sera until OD values reached background levels. Titers for sera that had background titers at the minimum dilution tested were less than 1:100 (<100).

little to no increase in anti-Env antibodies over the time period. In contrast, mice vaccinated with $sgp120_{IIIB}$-DNA or $sgp120_{IIIB}$-$mC3d_1$-DNA at weeks 0 and 6 and subsequently boosted with $gp120_{IIIB}$ protein at weeks 12 and 18 had a dramatic rise in anti-Env IgG (1:1,000). Non-DNA-primed mice that were vaccinated with $gp120_{IIIB}$ protein at week 12 showed no anti-Env antibodies in sera collected at week 14 (data not shown). These results indicate that mice that were primed with DNA during the first 12 weeks of vaccination had low antibody titers to Env but, when these mice were inoculated with purified $gp120_{IIIB}$ protein, they responded rapidly to vaccination. The range of anti-Env titers from sera collected at week 20 for each mouse group supports the conclusion that sgp120 fused to multiple copies of C3d elicits the highest antibody titers (Fig. 4).

A second set of DNA vaccine plasmids were constructed ($sgp120_{89.6}$, $sgp120_{89.6}$-$mC3d_3$, $sgp120_{ADA}$, and $sgp120_{ADA}$-$mC3d_3$) in order to compare the anti-Env antibody responses by using *env* genes from CCR5-utilizing virus isolates (Fig. 3C). HIV-$1_{89.6}$ is a dualtropic (R5X4) utilizing isolate and HIV-1_{ADA} is a macrophagetropic (R5) utilizing isolate. Each DNA plasmid was inoculated into BALB/c mice, and the induction of anti-Env antibody was compared to antisera from mice vaccinated with $sgp120_{IIIB}$-DNA or $sgp120_{IIIB}$-$mC3d_3$-DNA (Fig. 3C). All mice vaccinated with any of the sgp120-DNAs elicited no anti-Env antibody. In addition, mice vaccinated with DNA expressing the $mC3d_3$ only (tpA-$mC3d_3$-DNA) elicited no anti-Env or anti-$mC3d_3$ antibodies (Fig. 3C and data not shown). In contrast, mice vaccinated with any of the sgp120-$mC3d_3$-DNAs had increasing titers of anti-Env antibodies over the 20-week period. All mice had an antibody response to Env after the second vaccination which peaked at week 14 and maintained this titer for an additional 16 weeks (week 30). At week 20, mice vaccinated with sgp120-$mC3d_3$-DNA had an endpoint dilution titer of 1:5,000 (IIIB), 1:2,000 (ADA), and 1:1,000 (89.6).

Human C3d also enhances anti-Env antibodies. DNA plasmids expressing sgp120 fused to three tandem copies of the human homologue of C3d ($hC3d_3$) were constructed in a similar manner as the sgp120-$mC3d_3$ constructs described above. DNA vaccine plasmids used the *env* gene from the virus strains IIIB, 89.6, and JRFL. Each construct efficiently expressed the sgp120 or sgp120-$hC3d_3$ gene inserts (Fig. 5), and a 250-μg dose of each DNA was inoculated via intramuscular injection in 0.9% saline into the hind leg of a New Zealand White rabbit (Robinson Farms, Clemmons, N.C.). Rabbits were chosen to test sgp120-$hC3d_3$-DNA for enhancement of immune responses because 81% of the amino acid residues are identical (90% similar) between human C3d and rabbit C3d. However,

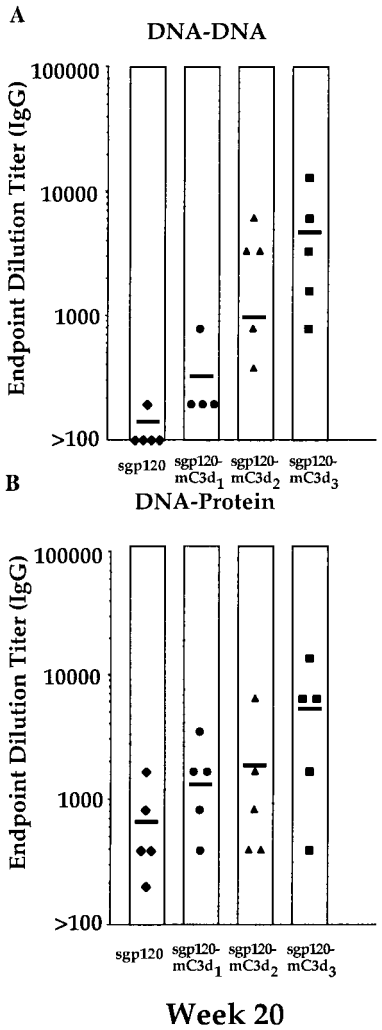

FIG. 4. Individual anti-Env IgG titer raised by DNAs expressing sgp120 fused to murine C3d. (A) 3. Mice were primed with DNA at day 0 and boosted at weeks 6, 12, and 18. Sera, collected at week 20, were assayed by ELISA for specific IgG, and the endpoint dilution titer was determined. (B) Mice were primed with DNA at days 0 and 6 and then boosted with $sgp120_{IIIB}$ at weeks 12 and 18. Then, 96-well plates were coated with recombinant gp120 protein-derived CHO cells expressing the HIV-1 isolate, IIIB. Each datum point represents the endpoint dilution titer for an individual mouse. Preimmune sera from mice had no detectable specific IgG. Endpoint dilution titers were conducted by diluting the sera until the OD values reached background levels. The horizontal line in each lane represents the averaged titer for that group. Other symbols for panels A and B are as defined in the legend to Fig. 3.

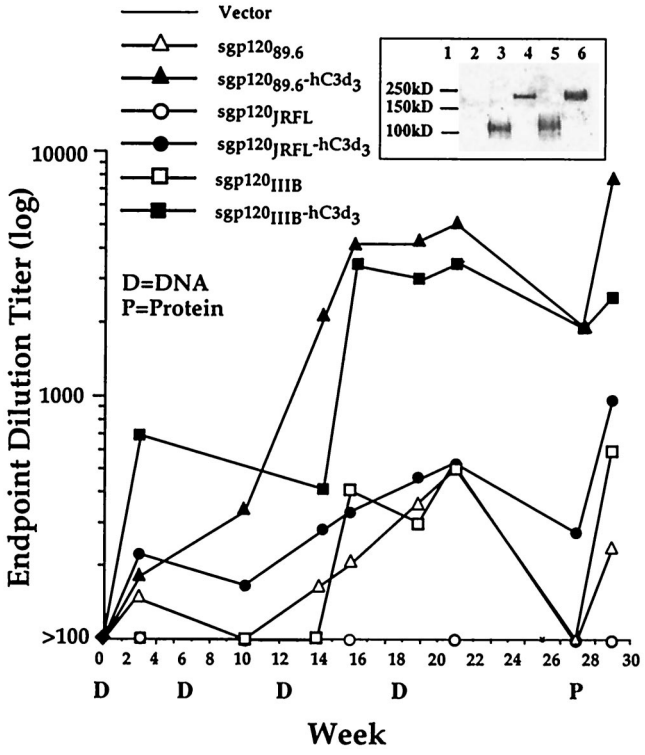

FIG. 5. Anti-Env IgG raised by intramuscular inoculation of DNAs expressing sgp120 fused to human C3d. Rabbits were primed with DNA at day 0; boosted at weeks 6, 12, and 18; and boosted with $sgp120_{IIIB}$ protein at week 27. Sera were obtained from rabbits at weeks 0, 6, 8, 12, 14, 18, 20, 27, and 29. Symbols: —, vector; △, $sgp120_{89.6}$-DNA; ○, $sgp120_{JRFL}$-DNA; □, $sgp120_{IIIB}$-DNA; ▲, $sgp120_{89.6}$-hC3d$_3$-DNA; ●, $sgp120_{JRFL}$-hC3d$_3$-DNA; ■, $sgp120_{89.6}$-hC3d$_3$-DNA. Sera were collected at the indicated times from each rabbit and assayed for specific IgG levels by ELISA. Then, 96-well plates were coated with recombinant gp120 protein-derived CHO cells expressing the HIV-1 isolate, IIIB. Data are the averages for five rabbits. Preimmune sera from mice had no detectable specific IgG. Endpoint dilution titers were conducted by diluting the sera until the OD values reached background levels. (Inset) Expression of vaccine constructs in vitro. Human embryonic kidney cells (293T) were transfected with 2 μg of each vaccine plasmid. Supernatant was collected, and 1.5% of total volume was subjected to electrophoresis on a 10% polyacrylamide gel. Lanes: 1, molecular mass marker; 2, tpA-hC3d$_3$; 3, $sgp120_{IIIB}$-DNA; 4, $sgp120_{IIIB}$-hC3d$_3$-DNA; 5, $sgp120_{JRFL}$-DNA; 6, $sgp120_{JRFL}$-hC3d$_3$-DNA.

71% of the amino acid residues are identical between human C3d and mouse C3d. In addition, human C3d bound to CR2 on the surface of rabbit B cells (Michael Steward [Adprotech], unpublished data). Therefore, rabbits were vaccinated at day 1 and boosted with the same DNA inoculum at weeks 6, 12, and 18. As seen with sgp120-mC3d fusions, DNA plasmids expressing any of the sgp120-hC3d$_3$ raised higher titers of anti-Env antibody than the sgp120-DNA (Fig. 5). An anti-Env endpoint dilution titer peaked as high as 1:8,000 ($sgp120_{89.6}$-hC3d$_3$) after the fourth vaccination. Rabbits vaccinated with any of the sgp120-DNA or tpA-hC3d$_3$-DNA elicited little or no anti-Env antibody. At week 27, all rabbits were vaccinated with 5 μg of affinity-purified $gp120_{IIIB}$ protein. The anti-Env titers in rabbits inoculated with $sgp120_{JRFL}$-hC3d$_3$-DNA, $sgp120_{89.6}$-hC3d$_3$-DNA, $sgp120_{IIIB}$-hC3d$_3$-DNA, or $sgp120_{89.6}$- $sgp120_{IIIB}$-DNA increased after protein vaccination (Fig. 5). The fold increase in elicited antibody between each matched sgp120-DNA and sgp120-hC3d$_3$-DNA varied between constructs. In our previous studies, differences in the levels of the antibody raised by the different Envs appeared to be determined by the specificity of the raised antibody (56). When antibody was captured on the homologous Env, all of the C3d fusions appeared to raise similar levels of antibody. In the present study, antibodies raised to R5 envelopes (particularly $sgp120_{JR\text{-}FL}$) did not have as high a titer on ELISA plates coated with purified $gp120_{IIIB}$

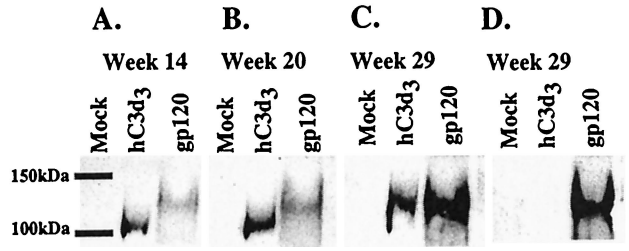

FIG. 6. Western blot of anti-$hC3d_3$ antibodies in sera from vaccinated rabbit. Human embryonic kidney cells (293T) were transfected with 2 μg of each vaccine plasmid. Supernatant was collected, and 1.5% of the total volume was subjected to electrophoresis on a 10% polyacrylamide gel. Each blot was probed with antisera from rabbits vaccinated with $sgp120_{89.6}$-$mC3d_3$-DNA at the indicated time points. (A) Week 14 antisera; (B) week 20 antisera; (C) week 29 antisera; (D) week 29 antisera mixed with 250 μg of purified $hC3d_3$ protein. Lanes: Mock, cell supernatant only; $hC3d_3$, tpA-$hC3d_3$; gp120, $sgp120_{89.6}$.

(1:1,000) as titers on plates coated with purified R5 gp120s (1:3,200). These results demonstrate that the human homologue of C3d, when fused to sgp120, enhances the antibody titer to Env.

Antisera from rabbits vaccinated with sgp120-$hC3d_3$-DNA were used to probe for Env and C3d protein. Sera collected at week 14, 20, and 29 detected $hC3d_3$, sgp120, and sgp120-$hC3d_3$ proteins by Western analysis (Fig. 6A to C). Sera from later time points produced more intense bands, indicating increasing titers of anti-Env and anti-$hC3d_3$ antibodies with each successive immunization. In order to determine the specificity of the rabbit sera for $hC3d_3$, antisera from rabbits vaccinated with sgp120-$hC3d_3$-DNA were incubated with $hC3d_3$ protein and then used to probe for proteins via Western analysis (Fig. 6D). Anti-$hC3d_3$-depleted antisera were unable to detect $hC3d_3$ protein but detected sgp120 Env protein. Therefore, anti-$hC3d_3$ antibodies are elicited in the rabbit more rapidly than anti-Env proteins; however, both antigen capture (Fig. 5) and Western analysis (Fig. 6) indicated that significant levels of anti-Env antibodies were elicited by DNA vaccination.

Avidity of anti-Env antiserum. Sodium thiocyanate (NaSCN) displacement ELISAs demonstrated that the avidity of the antibody generated with $sgp120_{IIIB}$-$mC3d_3$-expressing DNA was consistently higher than that from $sgp120_{IIIB}$-DNA-vaccinated mice (Fig. 7). Avidity assays were conducted on sera raised by $sgp120_{IIIB}$-DNA and $sgp120_{IIIB}$-$mC3d_3$-DNA because of the type specificity of the raised antisera and the commercial availability of the IIIB protein, but not the other proteins, for use as capture antigen. The avidity of specific antibodies to Env was compared by using graded concentrations of NaSCN, a chaotropic agent, to disrupt antigen-antibody interaction (48). The binding of antibodies with less avidity to the antigen is disrupted at lower concentrations of NaSCN than that of antibodies with greater avidity to the antigen. The 50% effective dose of NaSCN antiserum collected at week 20, after four inoculations of the $sgp120_{IIIB}$-$mC3d_3$-DNA vaccine, was ~1.75 M (Fig. 7) and was significantly higher than for mice vaccinated with $sgp120_{IIIB}$-$mC3d_1$-DNA ($P < 0.05$). Mice inoculated with two doses of DNA (weeks 0 and 6) and boosted with Env protein (weeks 12 and 18) had a

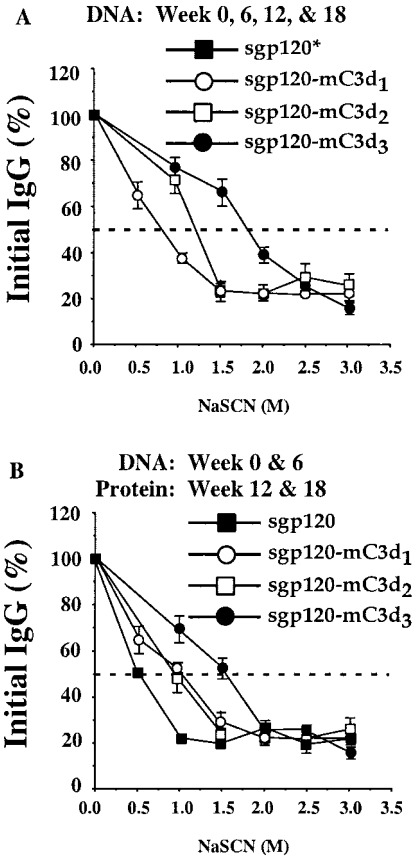

FIG. 7. Avidity of the anti-Env IgG raised by the IIIB Env-DNA vaccines. Sera were analyzed from week 20 in an Env-specific NaSCN-displacement ELISA. Plates were coated with recombinant $gp120_{IIIB}$. Sera were collected from $sgp120_{IIIB}$-DNA (■), $sgp120_{IIIB}$-$mC3d_1$-DNA (○), $sgp120_{IIIB}$-$mC3d_2$-DNA (□), and $sgp120_{IIIB}$-$mC3d_3$-DNA (●). Assays used pooled serum samples from each mouse group that was normalized to the same OD (range, 1:200 to 1:400 for $sgp120_{IIIB}$ and $sgp120_{IIIB}$-$mC3d_1$; range, 1:800 to 1:3,200 for $sgp120_{IIIB}$-$mC3d_2$-DNA and $sgp120_{IIIB}$-$mC3d_3$). Data are the averages of three independent experiments.

less dramatic response than mice inoculated with DNA expressing C3d-conjugated Env (Fig. 7). The avidity of the antisera from mice vaccinated with $sgp120_{IIIB}$-DNA was not measured, since no antibody was elicited (Fig. 3A). Antisera from mice primed with $sgp120_{IIIB}$-DNA and boosted with protein had an ED_{50} of ~0.5 M. However, mice vaccinated with DNA expressing sgp120-$mC3d_3$ had an ED_{50} of ~1.5 M. In addition, all mice vaccinated with sgp120-$mC3d_3$-DNA had higher ED_{50}s at week 14 (~1.15 M) compared to mice vaccinated with sgp120-DNA (~0.5 M). These results indicate that the antibody from $sgp120_{IIIB}$-$mC3d_3$-DNA-vaccinated mice had undergone more-rapid avidity maturation than antibody from sgp120-DNA-vaccinated mice.

Enhanced induction of neutralizing antibodies. Sera collected at week 20 from vaccinated mice were tested against two syncytium-inducing viruses, IIIB (X4) and 89.6 (R5X4), for virus-neutralizing activity. Sera from mice, primed with $sgp120_{IIIB}$-$mC3d_3$-DNA and boosted with Env protein, protected 50% of the MT-2 cells in vitro from $HIV\text{-}1_{IIIB}$ infection

TABLE 1. Neutralizing antibody titer from vaccinated mice and rabbits

Mouse group			Rabbit group	
DNA inoculum (wk 0 and 6)	DNA inoculum titer (wk 12 and 18)	$gp120_{IIIB}$ protein inoculum titer (wk 12 and 18)	DNA inoculum (wk 0, 6, 12, and 18)	$gp120_{IIIB}$ protein inoculum titer (wk 27)
$sgp120_{IIIB}$[a]	NT[c]	<1:20[b]	$sgp120_{IIIB}$[a]	<1:20[b]
$sgp120_{IIIB}$-$mC3d_1$	<1:20	1:27	$sgp120_{IIIB}$-$hC3d_3$	1:160
$sgp120_{IIIB}$-$mC3d_2$	1:36	1:33	$sgp120_{89.6}$	<1:20
$sgp120_{IIIB}$-$mC3d_3$	1:45	1:80	$sgp120_{89.6}$-$hC3d_3$	1:80

[a] Sera were tested against each homologous strain, and the titers are shown. Sera tested against the heterologous strain did not show neutralizing titers above background. The preimmune sera titer has been deducted from each sample.
[b] Titers are the sample dilution at which 50% of the MT-2 cells were protected from virus-induced killing as measured by neutral red uptake.
[c] NT, not tested.

at a dilution of 1:80 (virus-neutralizing titer) (Table 1). Sera from mice inoculated with four doses of $sgp120_{IIIB}$-$mC3d_3$-DNA elicited a neutralizing titer that protected 50% of the MT-2 cells from viral infection at a dilution of 1:45. Sera from mice vaccinated with DNA expressing $gp120_{IIIB}$ only did not elicit neutralizing antibodies that were detected in this assay (<1:20). In addition, there were no cross-reactive neutralizing antibodies to HIV-$1_{89.6}$ in the sera from mice vaccinated with any of the DNAs expressing sgp120 antigens, regardless of whether they were conjugated to C3d (data not shown).

Similar results were observed with rabbits vaccinated with DNA expressing C3d-conjugated vaccines. Neutralizing titers were observed in antisera collected at week 29 from rabbits primed with $gp120_{IIIB}$-$hC3d_3$ and boosted with purified $gp120_{IIIB}$ (≥1:80). Sera from these rabbits did not protect MT-2 cells from infection with HIV-$1_{89.6}$ (<1:20). Despite having higher IgG titers than sera collected from mice or rabbits vaccinated with $gp120_{IIIB}$-$C3d_3$-DNA, HIV-Ig did not elicit neutralizing antibodies that were detected in this assay (<1:20).

DISCUSSION

The C3d fragment of the third complement protein (C3) is one of the final degradation products of the complement system pathway (23). In mammalian immune systems, one consequence of complement activation is the covalent attachment of the C3d to an antigen, leading to the activation of the immune response (17, 18). In the present study, murine or human C3d was fused to a soluble form of the surface protein of the HIV-1 envelope in a DNA vaccine and tested in rodent systems for enhanced immunogenicity. Mice were vaccinated with DNA plasmids expressing sgp120 from the strain HIV-1_{IIIB} fused to different numbers of copies of murine C3d (one, two, or three copies). Mice vaccinated with DNA expressing sgp120 fused to multiple copies of C3d had higher titers of anti-Env antibodies compared to mice vaccinated with $sgp120_{IIIB}$-$mC3d_1$-DNA or sgp120-DNA (Fig. 3). Mice vaccinated with plasmids expressing sgp120 and $C3d_3$ on separate plasmids did not elicit detectable anti-Env antibodies after four inoculations (data not shown). These results are in agreement those of with Dempsey et al., who originally demonstrated that C3d must be fused to an antigen in order to elicit the enhanced immune responses to that antigen (19). Recently, Suradhat et al. reported that two copies of mC3d fused to bovine rotavirus (BRV) VP7 or bovine herpesvirus type 1 glycoprotein D did not enhance antibody titers to either antigen (61). However, in the present study, two copies of C3d fused to sgp120 elicited higher anti-Env titers than one copy, and three copies elicited higher anti-Env titers than two copies (Fig. 3). In addition, mice given two $sgp120_{IIIB}$-$mC3d_2$-DNA or $sgp120_{IIIB}$-$mC3d_3$-DNA inoculations, followed by two $sgp120_{IIIB}$ protein boosts, had anti-Env titers similar to those of mice vaccinated with four DNA inoculations (Fig. 3 and 4). However, in contrast, mice vaccinated with $sgp120_{IIIB}$-$mC3d_1$-DNA or sgp120-DNA elicited little antibody response with four DNA inoculations but did elicit detectable antibody titers when boosted with protein. The reason for the lack of an antibody response with VP7-$mC3d_2$ or glycoprotein D-$mC3d_2$ is unclear; however, the effectiveness of C3d appears to be antigen dependent. Recently, we have shown that when C3d is coupled to the HA of influenza virus, which is an immunogenic molecule compared to Env of HIV-1, the C3d enhancement of anti-HA titers may not be observed when higher doses of inoculum are administered (38, 55). In addition, the route of administration or the strain of mouse may influence the effectiveness of C3d fusion immunogens. Intramuscular inoculation of C3d vaccine plasmids into BALB/c mice consistently elicited lower titers to the fused antigen than did intradermal, gene gun inoculation of plasmids (38, 55). The CD21-mediated enhancement of B-cell activation is highly dependent on interleukin-4 (IL-4) production (39). Previous studies have indicated that IL-4 is enhanced in mice vaccinated with C3d-conjugated vaccines compared to non-C3d-fused forms (38, 56). Anti-$C3d_3$ antibodies were observed in rabbits but not in mice. We hypothesize that the production of anti-$hC3d_3$ antibodies in rabbits is due to the production of "non-self" $hC3d_3$ proteins in rabbits and, therefore, the lack of anti-$mC3d_3$ antibodies is expected in mice. Further experiments to address anti-$hC3d_3$ antibodies will have to wait until these vaccines are inoculated into human populations.

Direct stimulation of B cells, leading to cellular proliferation, has been proposed as a possible mechanism for C3d-mediated immune enhancement of antibody responses. The binding of C3d to CR2 (CD21) on the surface of circulating B cells has been shown to activate B cells (23). After C3d binds to the CR2, it cross-links with the signaling receptor, CD19; B cells then become activated and are directly stimulated to produce antibody (19, 22, 23). DNA expressing sgp120-$mC3d_3$ is likely to have supported the height of antibody responses by

(i) directly stimulating antibody-producing B cells through CR2 (CD21) and expanding the pool of anti-Env-specific B cells (22, 23), (ii) the possible lowering by C3d-conjugated antigen of both the concentration threshold and the affinity threshold for B-cell activation (14, 39), or (iii) reducing B-cell apoptosis (7, 31, 52).

CR2 has also been shown to augment antigen presentation by B cells. A previous study indicated that B cells that express higher levels of CR2 were more efficient in processing antigen than those with lower levels of CR2 (10, 52). Significant C3d-antigen immune complex binding requires a threshold level of CR2 expression, suggesting that only B cells with the highest levels of CR2 are likely to participate in the processing and presentation of antigens. C3d-coupled antigens in our vaccines may enhance the binding of low-affinity antigens and thereby enhance antigen presentation to T helper cells. The higher levels of IL-4 observed in mice vaccinated with $C3d_3$-coupled antigens may be a reflection of this enhanced presentation (38). Alternatively, C3d may extend the half-life of the antigen, allowing for improved presentation to the immune system. Future studies need to be performed to explore this possibility.

Immunization with DNA expressing sgp120 coupled to multiple copies of murine C3d resulted in enhanced avidity maturation of anti-Env antibody (Fig. 7). Previous studies have described the lengthy evolution of antibody responses to HIV infection (17, 29). Antibodies gradually mature over a period of months from low avidity to higher avidity levels (17, 29). Avidity maturation occurs in germinal centers where the somatic hypermutation of immunoglobulin results in a large repertoire of antigen-specific B cells that undergo selection for high-affinity B-cell receptors. $CR2^{-/-}$ mice have impaired humoral immunity, including decreased affinity/avidity maturation and germinal center development (68). C3d may enhance the avidity of the anti-Env response by binding to CR2 on FDC and thereby aiding the entry of B cells producing anti-Env antibody into germinal centers. Prior studies on the avidity of DNA-raised, anti-Env antibodies have also revealed low-avidity anti-Env antibodies (51). Env is a heavily glycosylated protein that may allow for it to remain "nonimmunogenic" in response to immune pressure during chronic HIV infection (6, 8). The formation of germinal centers, which are critical for antibody maturation, could be impeded by the glycosylation of Env (36, 63). C3d may help Env overcome this limitation by directing sgp120-C3d immunogens to FDC and enhancing germinal center formation, leading to enhanced avidity maturation. C3d may also assist in trafficking fused antigens to sites in the spleen where germinal centers form by binding to CD21 on FDC. Additional studies will be required to address the mechanisms of C3d enhancement and determine whether C3d is advancing germinal center formation in these vaccinated mice.

In contrast to the enhancement of antibody titers and avidity maturation of antibodies to Env, neither a DNA prime/DNA boost or a DNA prime/protein boost strategy with sgp120-C3d immunogens raised high titers of neutralizing antibody for HIV-1 Env (Table 1). Mice vaccinated with plasmids expressing sgp120-DNA had low levels of neutralizing antibody that were only modestly increased in mice vaccinated with sgp120-$mC3d_3$-expressing plasmids. In contrast to other studies (2, 50), priming with DNA followed by gp120 protein boosts did not increase the titer or breadth of neutralizing antibody. These differences could be attributed to the form of Env (monomers versus oligomers) used, the dose of DNA used, or the animal model (rodents versus primates) used for vaccination. Previous studies have suggested that oligomeric forms of Env are superior antigens for raising neutralizing antibody (24, 37, 50, 58, 65, 66). Different epitopes are exposed on oligomeric (gp140 or gp160) forms of Env compared to monomeric Env (gp120) (12, 41, 58). In addition, the ability of anti-Env antibodies to neutralize HIV-1 is predicted by binding to oligomeric but not monomeric forms of Env (9, 67). The intrinsic high background levels for HIV-1 neutralization assays in mouse sera may have contributed to the poor neutralization titers. The preimmune sera from these mice had anti-Env titers of 1:20 to 1:40 that were deducted from each of the postimmune serum titers. Additional vaccine studies with sgp120 fused to the human homologue of C3d were performed in rabbits, which do not show as high a background level in preimmune sera. Rabbits primed with DNA and boosted with protein or DNA showed higher neutralizing titers than did our mouse study.

The correlation between avidity maturation and neutralizing antibody production to gp120 has not always been clear. Some studies have indicated a good correlation (18, 32, 33), whereas others have not (16, 35). Future studies should be implemented with Env immunogens that can elicit cross-clade, neutralizing antibodies (9, 49, 57, 67), and therefore these titers can then be raised by coupling to C3d. In addition, future studies can explore the use of recombinant viral vectors (e.g., modified vaccinia virus Ankara [1, 3, 21, 25, 44, 45]), adenovirus 5 (15), or alphavirus (13, 64), which are being used in many preclinical AIDS vaccine studies. The introduction of C3d fused immunogens into these viral vectors could be a more effective strategy for enhancing immune responses.

One of the problems faced by those attempting to create an HIV/AIDS vaccine is the ability to protect against the diversity of isolates present in patient populations. A central goal has been to identify immunogens capable of raising high-titer, long-lasting, neutralizing antibody to the envelope glycoproteins of primary isolates. Our results presented in this study suggest that, once a suitable Env immunogen is identified for cross-reactive neutralizing antibody induction, C3d fusions might be helpful as a molecular adjuvant to enhance antibody production. If this can be accomplished, C3d fusions could have a major impact on the development of the neutralizing component for an AIDS vaccine. In addition, these results underscore the importance of increasing our understanding of the relationship between innate and acquired immunity. The use of C3d as a vaccine adjuvant has great potential for a variety of antigens, not only for infectious disease, including biodefense agents, but also for nonimmunogenic self-proteins.

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