Production and Characterization of High-Affinity Human Monoclonal Antibodies to Human Immunodeficiency Virus Type 1 Envelope Glycoproteins in a Mouse Model Expressing Human Immunoglobulins

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Received 14 July 2006/Returned for modification 11 October 2006/Accepted 28 November 2006

Human (Hu) monoclonal antibodies (MAbs) against the human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins (Env) are useful tools in the structural and functional analysis of Env, are under development both as potential prophylaxis and as therapy for established HIV-1 infection, and have crucial roles in guiding the design of preventative vaccines. Despite representing more than 50% of infections globally, no MAbs have been generated in any species against C clade HIV-1 Env. To generate HuMAbs to a novel Chinese C clade Env vaccine candidate (primary isolate strain HIV-197CN54), we used BAB5 mice that express a human immunoglobulin (Ig) M antibody repertoire in place of endogenous murine immunoglobulins. When immunized with HIV-197CN54 Env, these mice developed antigen-specific IgM antibodies. Hybridoma fusions using splenocytes from these mice enabled the isolation of two Env-specific IgM HuMAbs: N3C5 and N03B11. N3C5 bound to HIV-1 Env from clades A and C, whereas N03B11 bound two geographically distant clade C isolates but not Env from other clades. These HuMAbs bind conformational epitopes within the immunodominant region of the gp41 ectodomain. N3C5 weakly neutralized the autologous isolate in the absence of complement and weakly enhanced infection in the presence of complement. N03B11 has no effect on infectivity in either the presence or the absence of complement. These novel HuMAbs are useful reagents for the study of HIV-1 Env relevant to the global pandemic, and mice producing human immunoglobulin present a tool for the production of such antibodies.

Monoclonal antibodies (MAbs) are essential reagents in a wide variety of research, diagnostic, and clinical settings. MAbs against the human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins (Env) have enabled extensive studies into Env structure and antigenicity (25, 58, 60), and neutralizing MAbs (NMAbs) of human origin have been used therapeutically against HIV-1 in clinical trials (53) and as experimental micobicides in the simian-HIV macaque challenge model (54). Moreover, NMAbs are especially useful in the design of vaccine strategies that aim to recapitulate such neutralizing antibody (NAb) responses in vivo. Since infused human NMAbs provide sterilizing immunity against HIV-simian immunodeficiency virus chimeric viruses (SHIV) in macaque challenge models (13, 29, 45), such NAb responses elicited via active immunization might provide protective immunity against HIV-1 (7). Although an extensive catalogue of MAbs to HIV-1 Env currently exists (see http://hiv-web.lanl.gov/content/immunology/index.html for details), the majority of these were derived from patients infected with the B clade of HIV-1, which predominates in Europe and the Americas, or from animals immunized with Env from such isolates. Although many of these MAbs bind non-clade B Env, there are doubtless many Env epitopes that remain uncharacterized due to the focus on the B clade, which represents a minority of the circulating pandemic strains (21a). The C clade of HIV-1 accounts for 50% of infections globally and is particularly prevalent in sub-Saharan Africa and South Asia (21a). The relative “success” of C clade viruses justifies an intensified effort to understand their biology. To this end, the generation of MAbs should focus on clade C and other non-clade B Env isolates in order that the library of MAbs against HIV-1 Env can be expanded.

The production of MAbs often involves the use of rodents, as they are amenable to such studies in terms of costs and numbers, and their B cells are easily immortalized. However, the most useful MAbs in a clinical setting are human MAbs (HuMAbs), since they can be used clinically in humans without rejection by the host immune system. For this reason, many researchers have produced rodent MAbs and then made them suitable for use in humans by replacing portions of the rodent immunoglobulin (Ig) chain with the equivalent human structures. Such methodologies have allowed rodent MAbs to be used successfully in humans without induction of anti-rodent Ig Abs that neutralize the transferred rodent MAb (16). Obviously, making HuMAbs directly in rodents would obviate this...
genetic manipulation of the desired MAb clone. Moreover, all of the broadly reactive NMAbs against HIV-1 that have been derived to date are of human origin, and many, but not all, have long complementarity-determining region heavy chain 3 (CDR-H3) loops that mice and some other rodents are unable to produce (8, 10, 37, 41, 57). It is thought that these extended loops allow some NMAbs to access conserved, often recessed surfaces of HIV-1 Env and are thus responsible in part for their breadth of activity (7). Therefore, it has been recommended that Env-based vaccine antigens be tested in animal models, such as rabbits, that can elaborate long CDR-H3 loops and MAbs derived from nonhuman primates and humans infected with simian immunodeficiency virus and HIV-1, respectively (7). However, the development of transgenic mice producing human immunoglobulin provides a unique opportunity to isolate human MAbs from a rodent model, combining the convenience of rodents with the CDR-H3 loop flexibility of a human Ig repertoire. The BAB5 mice used in this study express a human IgM repertoire and are capable of producing affinity-matured Ab responses (35).

In the present study, we immunized BAB5 mice with the Env of strain HIV-1 

HIV-1 V3 loop, 447-52D (NIBSC, ARP3219, human, TCSN; S. Zoller-Pazner, New York University School of Medicine, New York, NY [17]), 196 (human, P. J. E. Robinson [43]), and Fab AH48 (human, P. D. R. Burton, The Scripps Research Institute, La Jolla, CA [67]) against glycans, 2G12 (human, P; H. Katinger and Robinson [43]), and Fab AH48 (human, P; D. R. Burton, The Scripps Research

MATERIALS AND METHODS

Reagents. Abs, MAbs, and other reagents were obtained from the following sources: the Centralised Facility for AIDS Reagents (supported by EU Programme EVA/MRC [contract QLKZ-CT-1999-00069]) and the UK Medical Research Council (NIBSC, Poetts Bar, United Kingdom) and the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Division of AIDS, NIAID). Otherwise, reagents were purchased from the indicated source or were donated directly by the producer and are listed with source and catalogue number (where appropriate), species, status, serum, purified [P], or concentrated tissue culture supernatant [TCSN]), and contributor in parentheses. Reagents used were as follows: HIV-1 Env C1 region, CA13 (NIBSC, ARP3119; mouse, P. C. Arnold); C1-4 regions, A32 (human; P. E. Robinson, Tulane Medical Centre, New Orleans, LA [30]); V2 loop, C180G (chimp; P. S. A. Tilley, Public Health Research Institute, New York, NY [56]); V3 loop, 447-52D (NIBSC, ARP3219, human, TCSN; S. Zoller-Pazner, New York University School of Medicine, New York, NY [17]), 196 (human, P. J. E. Robinson [43]), and Fab AH48 (human; P. D. R. Burton, The Scripps Research Institute, La Jolla, CA [67]) against glycans, 2G12 (human, P. H. Katunger and

G. Stiegler, Institute of Applied Microbiology, Vienna, Austria [3]; CD4 binding site, Gb1G12 (human, P. D. Burton and P. Parren [6]); CD4-induced (CD4d) surface (from J. E. Robinson except where indicated), ES1 (human, P [62], 412d (human, P [9]), 17b (human, P [51]), 48d (human, P [51]), and X5 (human, P; D. S. Dimitrov, CCRNR, CCR, NCI Frederick, NIH, Frederick, MD [33]); gp41 ectodomain, 5F3 (human, P; M. J. Frachette, Avens Pasteur, Marcy l’Etoile, France); 50-69 (human, P. S. Zoller-Pazner [18]), and 4E10 (NIH, 109F human, P. H. Katunger [49]); polyclonal Ab (PAB) against gp120 (ARP222, NIBSC, rabbit, serum, S. Ranjbar); Fab from seropositive patients to HIV Ig (human, P; NIH); gp120 C-terminal region-specific D7320 and p24-specific peptide (PAB) (Aalto Bio Reagents Ltd., Dublin, Ireland); human IgM(x) (Serorect, Oxford, United Kingdom); Ab conjugates, ARP 454 (NIBSC, mouse; P. B. Fern and R. Tedder and Dako Ltd., United Kingdom); goat anti-human IgM-horseradish peroxidase (HRP) and anti-human IgG-HRP (Jackson Immunoresearch Europe Ltd., Soham, United Kingdom); interleukin (IL)-2 (NIBSC, P; ARP901); soluble CD4 (scCD4) (NIBSC, ARP609, P); HIV-1 Env proteins and peptides gp140UG07, gp140h08, gp140o8z2, gp140oCN54, gp140oZM651s1, gp140oUG07G, and gp140oUG07 (P; S. Jeffs, Imperial College London, United Kingdom [23]); gp120h09 and gp120oCN54 (P; I. Jones, University of Reading, United Kingdom); gp120oCN54, NIBSC (ARP269, P, L. Williams); gp120oCN54 (NIBSC, EVA648, P, G. Voss); gp24oCN54, NIBSC (4961, P, M. Reitz); 15-mer peptide sets overlapping by 12 amino acids representing the gp120 portion of gp140oCN54 (EuroVacc and G. Pantaleo, Lausanne, Switzerland) or the whole of the gp140oCN54 ectodomain, amino acids 499 to 680, including an overlap of the gp120-gp41 junction and the start of the transmembrane domain (Pepscan Systems BV, Lelystad, The Netherlands).

Mice. Ten- to twelve-week-old BAB5 mice were obtained from the joint stock of the free-living non-human primate facility of the MRC Laboratory of Molecular Biology and the Babraham Institute (Cambridge, United Kingdom). BAB5 mice carry the human Ig heavy and both κ and λ light chain loci and have inactive endogenous Ig heavy and κ loci (35). All experiments were performed under appropriate licenses in accordance with the UK Animals (Scientific Procedures) Act 1986.

Immunizations and preparation of hybridomas. The mice were immunized by subcutaneous injection of 25 μg of gp140oCN54, formulated with 15 μl of the CpG-oligodeoxynucleotide based ImmuneAdjuvant (QIAGEN Ltd., Crawley, United Kingdom). In all, four booster immunizations were given, and blood samples were taken from the tail for serological analysis immediately before each immunization. After the final booster immunization the mice were transferred to a licensed animal facility at the University of Oxford, where they were euthanized 3 days after the final booster and the spleenocytes were harvested for the preparation of hybridomas. Hybridomas were prepared by the fusion of the immune splenocytes isolated from immunized BAB5 mice with NS-1 murine myeloma cells using polyethylene glycol 1500 (Roche Diagnostics, Lewes, United Kingdom). In all, four booster immunizations were given, and blood samples were taken from the tail for serological analysis immediately before each immunization. After the final booster immunization the mice were transferred to a licensed animal facility at the University of Oxford, where they were euthanized 3 days after the final booster and the spleenocytes were harvested for the preparation of hybridomas. Hybridomas were prepared by the fusion of the immune splenocytes isolated from immunized BAB5 mice with NS-1 murine myeloma cells using polyethylene glycol 1500 (Roche Diagnostics, Lewes, United Kingdom) with a 10-kDa molecular weight cutoff against sterile phosphate-buffered saline (PBS) and 20% fetal calf serum (FCS). The supernatants from the hybridomas were screened for reactivity with gp140oCN54 and a second HIV-1 clade C Env antigen, gp140oZM651, by enzyme-linked immunosorbent assay (ELISA). A selection of 40 clones was also used to screen gp120oCN54 using the same ELISA. Selected colonies underwent four rounds of dilution cloning before a final round of colony selection based on MAB production levels.

Production and concentration of IgM MAbs. Clarified hybridoma TCSNs were filtered using a 0.22-μm-pore-size Stericup filter (Millipore Ltd., Watford, United Kingdom). Sterile ammonium sulfate solution (76% wt/vol of (NH4)2SO4; Merck Chemicals Ltd., Poole, United Kingdom) was added in the ratio 1:1 (vol/vol) to the filtered TCSN, the mixture was incubated at 4°C for 2 h and centrifuged at 13,000 × g for 1 h at 4°C, and the pellet was dissolved in sterile distilled H2O. The precipitation process was repeated, and the solution was then dialyzed in SnakeSkin tubing (Perbio Science UK Ltd., Cramlington, United Kingdom) with a 10-kDa molecular weight cutoff against sterile phosphate-buffered saline (PBS) at 4°C. Dialyzed IgM MAbs were filter sterilized using a 0.22-μm-pore-size Spin-X filters (Fischer Scientific UK, Loughborough, United Kingdom). The IgM MAb concentration was determined by ELISA with known concentrations of human IgM(e) as a standard.

ELISAs. ELISA plates (Greiner Bio-One Ltd., United Kingdom) were coated with 50 μl/well of gp140oCN54 at 1 μg·mL−1 or any of the nested gp140oCN54 peptides at a concentration of 10 μg·mL−1, in 100 mM NaHCO3 (pH 8.5) overnight at 4°C. The plates were washed three times in phosphate-buffered saline (PBS) supplemented with 0.05% Tween 20 and blocked for 1 h at room temperature with 200 μl/well of 2% (wt/vol) nonfat milk (Marlval) dissolved in PBS supplemented with 0.05% Tween 20. Plates were washed as before, and a dilution series of either IgM MAb or TCSN in PBS containing 1% (wt/vol) bovine serum albumin (BSA) (as sample buffer [SB]) was added directly to the ELISA plate for 1 h. For the gp140oCN54 capture ELISAs, plates were coated with 1:2 to 1:10
(vol/vol) diluted IgM MAb containing TCSN. After blocking, a gp140\textsubscript{97CN54} dilution series in SB was added for 2 h. MAb IgG1b12 or antisera AR422 was used to detect captured gp140\textsubscript{97CN54}. For the MAb competition ELISAs, antigens was incubated with a saturating concentration of the first MAb for 1 h before the addition of a 50% binding concentration of the second MAb and incubation for 1 h more. After washing, 50 \mu l of the appropriate HRP-conjugated anti-species IgG or IgM Ab at a concentration of 0.8 \mu g·ml\textsuperscript{-1} diluted in SB was added for 1 h. ELISAs were then developed using TMB reagent (Jencons, United Kingdom), and the absorbance was measured at 450 nm. Assay cutoffs were calculated as the mean absorbance plus two standard deviations of wells that lacked primary Abs but were otherwise treated identically. The binding of the MAb in the presence of an excess amount of competing MAb was compared to the binding of the MAb without competing MAb.

Size-exclusion chromatography. Size exclusion chromatography was performed using a purifier (AKTApurifier; Amersham Biosciences Ltd.) with a Superose 6 HR 10/30 column (Amersham Biosciences Ltd.). Separation was performed at room temperature using a flow rate of 0.3 ml·min\textsuperscript{-1} with the combinations of CD4i surface MAbs and sCD4. All experiments were carried out at 25°C. Any signal detected in the BSA flow cell was subtracted from the MAb-specific signal.

Surface plasmon resonance (SPR). Twelve thousand response units of gp140\textsubscript{97CN54} and an irrelevant control protein, BSA (Sigma-Aldrich), were immobilized onto sensor chip CM5 flow cells (Biacore) as described previously (2) using the BIACore 2000 (Biacore, Upsalla, Sweden). MAbs and sCD4 at a concentration of 50 \mu g·ml\textsuperscript{-1} (except 447-52D, which was used at 10 \mu g·ml\textsuperscript{-1}) were injected into the flow cells. For competition analysis MAbs were used at saturating concentrations. The CD4i-specific MAbs and A32 were tested alone or in combination with sCD4. The sCD4 alone was injected over the chip surface through the flow-cell pathway, and the mean response was subtracted from the response observed with the combinations of CD4i surface MAbs and sCD4. All experiments were carried out at 25°C. Any signal detected in the BSA flow cell was subtracted from that in the gp140\textsubscript{97CN54} flow cell to give the antigen-specific signal.

Determination of relative avidity or true affinity by ELISA. To determine the relative avidity of the concentrated IgM MAbs, binding to gp140 was then measured in a standard ELISA. The relative avidity is reported as the concentration of MAb that gave half-maximal binding. The dissociation constant (K\textsubscript{D}) was calculated for the monomeric fractions of the MAbs by the methods described elsewhere (14, 44, 48). Each MAb was tested in triplicate, the experiment was repeated twice, and the average K\textsubscript{D} was then determined from the pooled data.

Neutralization and Ab-dependent complement-mediated virus inactivation assays. The IgM MAbs were tested for neutralizing activity and complement-mediated inactivation (CMI) against primary isolate HIV-1\textsubscript{97CN54} (gift from C. Moog, INSERM, Strasbourg, France) (50) in an infectivity reduction assay using phytohemagglutinin-HL-2-activated human peripheral blood mononuclear cells. For the CMI assay a 10% final concentration of fresh normal human serum (NHS) was included as a source of complement. As a control, serum from the same donor that had been heat inactivated (HI) at 56°C for 30 min was used.

RESULTS

The antigenic profile of gp140\textsubscript{97CN54}. Native HIV-1 Env has a complex quaternary structure that should be retained in the soluble form of the glycoprotein if NMAbs to conserved, discontinuous epitopes are to be obtained after immunization. Real-time SPR analysis of intermolecular interactions allows detection of weak interactions and those with fast dissociate rate constants that can go undetected by ELISA. We therefore characterized the antigenicity of gp140\textsubscript{97CN54} by SPR using 15 MAbs, 2 PAbs, 1 oligoclonal Ab (raised against a gp120 peptide), and sCD4 (Fig. 1A and B) to evaluate whether the molecule is appropriately folded. gp140\textsubscript{97CN54} bound sCD4 and the antibody 412d, whose gp120 binding characteristics closely mimic those of the N terminus of the coreceptor CCR5 (61), suggesting that the molecule is capable of binding both its receptors. Of the five CD4i-specific MAbs tested, two bound to gp140\textsubscript{97CN54} in the absence of sCD4 while all five bound when sCD4 was cojected (Fig. 1B). MAbs E51 and 412d demonstrated a similar binding profile, showing some binding in the absence of sCD4, which increased four- to fivefold upon coinjection of sCD4 (P = equal to 0.0286 in each case; one-tailed Mann-Whitney test). Binding of MAbs 48d, 17b, and X5 was only observed in the presence of sCD4, consistent with the phenomenon of coreceptor binding site exposure after engagement of sCD4 and indicative of a functional Env molecule. C1-C4 region-specific MAb A32 bound in the absence of sCD4 as expected (59), and binding increased by 40% when sCD4 was cojected (P = 0.0286; one-tailed Mann-Whitney test).

Overall, gp140\textsubscript{97CN54} was recognized by 13 of 15 MAbs and 2 of 3 PAbs despite being of clade C origin. The panel of Abs used here were derived from humans infected with clade B virus or animals immunized with clade B Env, except for CA13, which was derived from a mouse vaccinated with a clade A construct. The recognition of conformational epitopes involved in CD4 binding (IgG1b12) and of the CD4i surface of gp140\textsubscript{97CN54} suggests that this recombinant antigen has a native structure. Of the moderately to broadly reactive NMAbs, IgG1b12 and 447-52D bound gp140\textsubscript{97CN54} but 2G12 did not, as the glycan epitope is absent.

HIV-1\textsubscript{97CN54} Env does not possess the epitope for the broadly reactive NMAb 2FS, and the gp140\textsubscript{97CN54} construct terminates just before the epitope for the broadly active NMAb 4E10, which is otherwise present on the HIV-1\textsubscript{97CN54} virus. The degree and stability of binding varied greatly depending on the Ab and the nature of the epitope involved (Fig. 1C and D). IgG1b12 and 447-52D bound with relatively high avidity but dissociated substantially within 420 s of the end of MAb injection. Similarly, sCD4 dissociated noticeably from the oligomer (Fig. 1C). By contrast, MAb 5FS and PAb HIV1g both bound with high avidity and did not dissociate appreciably in the 420 s after injection (Fig. 1D).

Immune response to gp140\textsubscript{97CN54} in BAB5 mice. The immunogenicity of gp140\textsubscript{97CN54} in BALB/c mice has been investigated and shown to elicit reciprocal endpoint IgG titers on the order of 105 when injected subcutaneously in the presence of CpG-oligodeoxynucleotide-containing adjuvants (data not shown). Because BAB5 mice generally produce lower titers of Ab in response to immunization than nontransgenic animals (35), we gave the mice booster injections four times to achieve maximal responses and to encourage affinity maturation. Three BAB5 mice were immunized a total of five times with 25-\mu g doses of gp140\textsubscript{97CN54} (Fig. 2), and their antigen-specific IgM titers were established after one, two, four, and five immunizations. IgM titers were detected after a single immunization of gp140\textsubscript{97CN54}, and these increased by approximately fourfold after a booster immunization. On the day of the fusion the
reciprocal titers were ~300. Titers of this magnitude are in keeping with those seen after immunization of BAB5 mice with other antigens (38), and moreover this final bleed was taken 3 days after the fifth booster was given, too early for detection of peak serum Ab titers. Other studies have suggested that MAbs can be readily isolated despite relatively weak serum Ab responses (55). Due to the low IgM titers on the day of the fusion and the limiting quantities of serum, the sera were not tested for neutralization of HIV-197CN54.

Isolation and characterization of the multimeric nature of IgM MAbs after hybridoma fusion. Of the three BAB5 mice that were immunized, the splenocytes from one mouse were fused with NS-1 myeloma cells on the day of culling while the others were cryopreserved. In the initial fusion, 77 primary hybridomas were obtained, of which 14 showed significant reactivity with gp14097CN54 and 2 reacted with gp14096ZM651.8. Thirteen of the hybridomas either failed to grow or grew but lost antigen reactivity during dilution cloning. Several attempts to produce hybridomas from the cryopreserved splenocytes were unsuccessful. Eventually a second successful fusion from a different mouse produced 42 primary hybridomas, of which 11 showed reactivity towards gp14097CN54 and 1 showed reac-
activity towards gp140<sub>96ZM651.8</sub>. Again, many hybridomas failed to grow or progressively lost antigen reactivity. Finally, two IgM MAbs, N3C5 and N03B11, were isolated from two of the three mice. These clones stably secreted IgM MAbs and were shown by ELISA to capture gp140<sub>97CN54</sub> which could then be detected using MAb IgG1b12. Both novel MAbs were shown to use light chains (data not shown). N3C5 and N03B11 were concentrated by ammonium sulfate precipitation to yield stocks of approximately 200 μg · ml<sup>-1</sup> (N3C5) and 4 μg · ml<sup>-1</sup> (N03B11). To ascertain whether the IgM MAbs were pentameric or monomeric IgM, the two forms were separated by size exclusion chromatography (Fig. 3), and the eluate fractions were tested by ELISA to determine which contained IgM. Both IgM MAbs consisted of a mixture of pentamers and monomers as shown by the spread of human IgM in the fractions between gp140<sub>97CN54</sub> and gp140<sub>180kDa</sub>. Considerable running of one species into subsequent fractions occurred, as is typical with this technique. The monomers outnumbered pentamers by approximately 10:1 in pooled fractions covering the separate peaks (data not shown). The monomer and pentamer fractions were tested for binding to gp140<sub>97CN54</sub> (Fig. 4); both forms bound gp140<sub>97CN54</sub> when applied at equal concentrations. Under these conditions an equal number of binding sites were present, but there were fivefold fewer pentameric than monomeric molecules. Monomeric N3C5 bound to gp140<sub>97CN54</sub> to a greater extent than the same MAb in pentameric form (P = 0.0001; unpaired two-tailed t test), suggesting that steric factors may render the binding of bulky pentamers less favorable than the binding of the monomeric form. N03B11 monomers and pentamers appeared to bind equally in this ELISA.

**Breadth of recognition, relative avidity, and true affinity of N3C5 and N03B11.** We estimated the relative binding avidity of the ammonium sulfate concentrated MAbs for a panel of Env by ELISA, to establish the conservation of the epitopes for these MAbs among isolates from clades A, B, C, D, and F (Fig. 5). MAb N3C5 bound the homologous gp140<sub>97CN54</sub> with high avidity and bound heterologous gp140s from clades A and C with similar or slightly lower avidity, whereas N03B11 bound to the homologous gp140 and another gp140 from a geographically distant clade C isolate. Neither of the MAbs bound to gp120<sub>97CN54</sub> nor to a range of other gp120s from clade B (data not shown), suggesting that the epitope was either trimer dependent or located on the gp41 ectodomain. The avidity of N3C5 for the homologous antigen was significantly higher than that of N03B11 (median avidity of 0.27 nM versus 1.5 nM; P = 0.0012; two-tailed Mann-Whitney test with Bonferroni’s correction). However, the binding avidity did not differ significantly when the two MAbs were compared on gp140<sub>96ZM651.8</sub>-N3C5 bound significantly better to gp140<sub>97CN54</sub> than to gp140<sub>96ZM651.8</sub> (0.27 nM versus 1.0 nM; P = 0.0008; two-tailed Mann-Whitney test with Bonferroni’s correction), but the binding of N3C5 and N03B11 did not vary significantly between gp140<sub>97CN54</sub> and gp140<sub>96ZM651.8</sub>.

The KD of the monomeric fraction was tested at 22 and 37°C at two solid-phase antigen concentrations (Fig. 6). Insufficient quantities of pentamers were obtained to determine the KD for this fraction. The results show that an increase in temperature from 22°C to 37°C had no significant effect on the KD at either concentration of solid-phase antigen. The 50-fold change in solid-phase antigen concentration had no significant effect on...
the $K_D$ of either MAb on gp140$_{97CN54}$ at either temperature. However, at 37°C the $K_D$ of N3C5 on gp140$_{96ZM651.8}$ differed 2.2-fold for a 50-fold difference in coating antigen concentration ($P = 0.0076$; two-tailed unpaired $t$ test with Bonferroni’s correction). The binding of N3C5 to gp140$_{92UG037}$, known to be weaker than its binding to the clade C Env molecules from the earlier experiment (Fig. 5), could not be detected at a solid-phase concentration of 0.1 $\mu$g·mL$^{-1}$. Overall, at 22°C, coating antigen concentration had no significant effect on the calculated $K_D$ for either MAb on either clade C gp140. Unlike the relative avidity assay (Fig. 5) which used the crude MAb preparations, this assay showed that at 22°C and with a solid-phase antigen concentration of 5 $\mu$g·mL$^{-1}$, N3C5 had a higher affinity for gp140$_{96ZM651.8}$ than gp140$_{92UG037}$, with 96-fold ($P = 0.0066$) and 60-fold geometric mean differences ($P = 0.0285$), respectively (two-tailed Mann-Whitney test with Bonferroni’s correction). In addition, N3C5 had a higher affinity for gp140$_{96ZM651.8}$ than N03B11 did (140-fold geometric mean difference; $P = 0.0066$; two-tailed Mann-Whitney test with Bonferroni’s correction). The affinities of N3C5 and N03B11 for the various gp140 molecules are listed in Table 1. The first assay is useful in that it reflects the probable outcome when the MAbs are used in a standard ELISA, but it cannot be compared directly to the affinity assay due to important differences between the reagents, assays, and calculations.

Characterizing the epitopes of N3C5 and N03B11. The studies with the panel of Env, including gp140 and gp120 molecules, suggested that both MAbs bound to the gp41 region. To explore the epitope further, both MAbs were tested for binding to a set of linear 15-mer peptides representing the entire gp140$_{97CN54}$ sequence. Whereas MAbs 447-52D and 4E10 recognized their specific peptides in the V3 loop and membrane-proximal extracellular region (MPER), respectively, the IgM MAbs did not bind to the linear peptides (data not shown). To determine whether the epitopes of N3C5 and N03B11 were linear or conformational in nature, the binding of both MAbs to native and denatured Env was examined by ELISA (Fig. 7A). Binding of the MAbs to both native and denatured gp140$_{97CN54}$ was detectable, but binding to the denatured mol-
To narrow down the region containing the N3C5 and N03B11 epitopes, both MAbs were studied in competition analyses against gp41 MAb 5F3 (3) and 50-69 (18) and against each other (Fig. 7B; see Fig. S1 in the supplemental material for the positions of the 5F3 and 50-69 epitopes in relation to the structural features of the gp41 ectodomain). These studies showed a high degree of concordance between results from SPR assays and ELISA. MAb 5F3 inhibited the binding of N3C5 by 80 to 90% and of N03B11 by 98 to 100%. When the reciprocal competition experiments were performed, N3C5 inhibited the binding of 5F3 by 21 to 42%, whereas N03B11 inhibited 5F3 binding by only 8 to 15%. MAb 50-69 inhibited the binding of N3C5 in the SPR assay by 25% but did not significantly inhibit N3C5 when measured in the ELISA assay. The inhibition of N03B11 by 50-69 was more consistent in both assays, at 43% and 61% in ELISA and the SPR assay, respectively. In the reciprocal competition experiments, N3C5 inhibited the binding of 50-69 by SPR but not in the ELISA assay, whereas N03B11 did not inhibit 50-69 to a significant degree in either assay. The two IgM MAbs were tested against each

![Graph](image-url)
other by SPR only, and it was found that N3C5 inhibited the binding of N03B11 by 61%, while N03B11 did not significantly inhibit the binding of N3C5. The binding of controls, the MAb IgG1b12 (to a conformational epitope in the CD4 binding site), and the polyclonal antibodies ARP422 and HIV Ig was not affected significantly by any of the gp41 ectodomain region-specific MAbs (data not shown). Taken together, these results suggest that N3C5 and N03B11 both have conformational epitopes in the gp41 ectodomain proximal to the 5F3 and 50-69 epitopes, overlapping the gp41 immunodominant region. N3C5 and N03B11 are unlikely to share the same epitope due to differences in their ability to block the binding of one another and MAb 50-59, as well as differences in avidity of binding to gp140/52D.

A multiple sequence alignment of the gp41 ectodomains of isolates 97CN54, 97ZM651.8, 92UG037, 93BR029, H1B, HAN2, and 92UG021 (see Fig. S1 in the supplemental material) showed that there were 24 amino acid substitutions between the clade C isolates that bound N03B11 and the clade A isolate that did not. These included 8 nonconservative, 6 semiconservative, and 10 conservative substitutions. These were scattered throughout the gp41 ectodomain but were more frequent on the C-terminal side of the 50-69 epitope (five of seven substitutions).

**Effects of complement on MAb neutralization of HIV-1**

To determine whether the IgM MAbs possess antiviral activity, we tested their ability to reduce the infectivity of the autologous primary isolate HIV-1_97CN54, or in the presence of fresh NHS as a source of complement. At a concentration of 100 μg · ml⁻¹ N3C5 demonstrated weak but statistically significant neutralizing activity, reducing the viral titer by 79% (P < 0.05; unpaired one-tailed t test), while N03B11 reduced viral titer by 59%, but this was not statistically significant (Fig. 8). By comparison, the positive control MAb 447-52D achieved 92% neutralization at a concentration of 10 μg · ml⁻¹. Interestingly, when N3C5 was combined with fresh NHS, the degree of neutralization was half that seen with the combination with HI NHS, (P < 0.05; unpaired one-tailed t test). N03B11 appeared to inhibit infection in the presence of complement, but this result was not significant. The fact that neither MAb is potently neutralizing is not surprising given the paucity of NAb responses against primary HIV-1 isolates generated to vaccine candidates tested to date and given the fact that with the exception of the MPER, the gp41 ectodomain is largely obscured by gp120 on virion-associated Env.

**DISCUSSION**

Here we describe the use of mice producing human Ig to derive human IgM MAbs to a novel clade C HIV-1 Env vaccine candidate. This study demonstrates proof of principle for the use of such transgenic mice for the direct preparation of HuMAbs to HIV-1 Env-based vaccine antigens. While these mice might be used to produce novel HuMAbs, their utility in preclinical HIV-1 vaccine studies for screening of vaccine antigens may be restricted. The chief reason for this is the low titers of serum Abs (35) and the low volumes of serum that can be obtained for neutralization studies. Since neither IgM MAb reported here binds to representative clade B isolates, this suggests that these unique MAbs may be useful probes in further studies of the antigenicity of non-clade B Env and may also have utility in a clade-specific diagnostic setting. Since the human Ig-producing BAB5 mouse strain used here produces high-affinity IgM MAbs as opposed to IgG MAbs, these MAbs would be suitable as capture Abs when assessing human IgG-binding Ab responses. Other potential applications include use in Ab competition studies and as affinity chromatography reagents to purify Env.

The predominantly monomeric nature of N3C5 and N03B11 is likely to be due to the inability of the murine J chain to substitute adequately for the human J chain. Despite this and the lack of class switching from IgM to IgG in these mice, the IgM response underwent efficient affinity maturation as demonstrated by the derivation of the high-affinity MAbs N3C5 and N03B11. Prior studies using human Ig-producing transgenic mice have demonstrated their suitability for deriving MAbs against antigens such as human blood cells, tumor cell lines, haptons, the human acetylcholine receptor, and HIV-1 Env antigens (20, 21, 28, 35, 55). Interestingly, the immunization of the XMG2 XenoMouse strain with gp120SF162 allowed the isolation of IgG2(κ) MAbs that displayed neutralizing activity against the autologous primary isolate HIV-1SF162.
known to be relatively susceptible to neutralization, in a sensitive pseudovirus assay (21). The same mouse strain was also used to derive 39 MAbs to the full-length single-chain analogue of the gp120-CD4 receptor complex, composed of gp120\_{Ba-L} and domains 1 and 2 of CD4. However, none of this panel of MAbs possessed neutralizing activity against HIV-1\_{Ba-L} or HIV-1\_{39SF162}, potentially underlining the insufficiencies of the immunogen as an HIV-1 vaccine candidate (20). We have taken the derivation of human MAbs in transgenic mice further in our model system by the use of a more sophisticated trimERIC gp140 immunogen from a highly relevant primary clade C isolate that we demonstrated to be functional and appropriately folded. As far as we are aware, this is the first publication regarding MAbs of any species induced to clade C isolate. This finding suggests that N3C5 cannot bind the functional virion-associated Env with a similarly high affinity. It is known that the ability of MAbs to bind virion-associated Env is necessary but not sufficient for neutralization (4, 32, 36), while the ability to bind any epitope on a functional spike is a dominant determinant of neutralization (64). Indeed, many nonneutralizing MAbs appear to bind nonfunctional forms of virion-associated Env; the most important of these have been identified as gp120-gp41 monomers (32) and gp120-depleted gp41 stumps (4). However, other forms, including the uncleaved gp160 precursor, alternative trimERIC isoforms that expose the nonneutralizing face of gp120, or gp120-gp41 dimers or tetramers, may also serve as decoys (32). Since N3C5 was raised specifically against a trimeric uncleaved gp140, the most likely explanation for its failure to neutralize the virus potently is that the epitope is well-exposed on the trimeric recombinant uncleaved form but is either partially occluded or that it is conformationally altered on the cleaved, virion-associated form. Structural alterations in the epitope may arise from cleavage at the gp120-gp41 cleavage site, interactions between the MPER and the viral lipid envelope, or may depend on an intact intraviral gp41 tail, the presence of which has been demonstrated to modulate epitope exposure and viral neutralization sensitivity (22, 24). These potential differences in epitope conformation between the immunogen and the virion-associated Env could result in unfavorable binding kinetics between N3C5 and the virus. These could include a slow on rate or fast off rate, thus reducing neutralization potency (47). Moreover, other MAbs to this region are generally not neutralizing (15, 63, 66) or, similar to N3C5, are weakly neutralizing (11, 12), suggesting that this may be a general phenomenon associated with this region of Env. If steric occlusion of MAb binding is responsible for the weak neutralization activity of N3C5, then Fab fragments may be more potent inhibitors, in a manner analogous to that observed with CD4-induced epitope-binding MAbs such as 17b and X5 (26). The fact that N3C5 was weakly neutralizing but enhanced HIV-1 infection in the presence of human complement reinforces the concept that this MAb binds virion-associated Env, although gp41 stumps present a potential target for this MAb on the virion that would allow it to bind and activate complement without neutralizing virus infectivity. Moreover, MAbs to epitopes defined as gp41 cluster I and cluster II enhanced infection in the presence of complement in a manner similar to that observed with N3C5 (39). The inability of N3C5 to mediate CMI may be due to its monomeric nature. Pentameric IgM is a potent activator of the lytic complement cascade, and if more of the MAb had been in the pentameric form and this was not sterically occluded from the epitope, we might have observed CMI. Instead, it is likely that binding of monomeric IgM led only to complement-mediated opsonization followed by increased infection of target cells expressing complement receptors, such as macrophages (40).

Our attempts to define the epitope of these MAbs lead us to conclude that both MAbs bind distinct but related conformational epitopes in the immunodominant region of the gp41 ectodomain. Interestingly, neither MAb we isolated here bound to the two clade B gp140 molecules we tested, indicating a lack of epitope conservation in B clade viruses. Moreover, MAb N3C5 bound to the geographically distant heterologous clade C strain gp140\_{SFM12} (a Zambian isolate) with an affinity almost 1 log_{10} fold higher than that of binding to the autologous isolate, suggesting that gp140\_{SFM12} presents the epitope in a more favorable context. A related study comparing the binding of a panel of MAbs to eight clade C gp140s has indicated that gp140\_{SFM12} is recognized by the widest range of MAbs with the strongest affinities, while gp140\_{SFM4} shows a less favorable antigenic topology (S. A. Jeffs, S. Larsen, and S. M. Vieira, unpublished results). In all, 24 amino acids varied between isolates that bound N03B11 and those that did not. Only seven amino acids varied between strains that bound N3C5 and those that did not. Although we cannot rule out the possibility that different substitutions affected the binding in different ways, it is likely that the critical contact residues are among those identified.

In conclusion, we describe the isolation from transgenic mice immunized with clade C HIV-1 gp140 of two human MAbs, one of which is weakly neutralizing for the autologous virus. We propose that such mice might be useful for the future production of HuMAbs against antigens of interest and that the novel MAbs obtained will be of use as probes for Env antigenicity and function.

ACKNOWLEDGMENTS

We acknowledge the support of the EU Consortium Eurovac and the UK Medical Research Council (grant G0008035). S.L.D. was supported by a grant from the AICR to M. S. Neuberger. We thank M. Brüggemann and Michael S. Neuberger for provision of the BAB5 mice; J. E. Robinson, D. R. Burton, D. Katinger, and S. Zolla-Pazner for their generous gifts of MAbs; G. Pantaleo and Eurovac for CN54 gp120 sequence-derived synthetic peptides; and the NIBSC CFAR and NIH Reagent Program for reagent supply. We also thank C. Moog for providing HIV-1\_{39CN54}, M. Puklavec for providing the NS-1 murine myeloma cells, and A. Akoulitchev for assistance with the liquid chromatography. We have no conflicting commercial interests relevant to the work published here.
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