JOURNAL OF VIROLOGY, Dec. 1998, p. 9656–9667 Vol. 72, No. 12
0022-538X/98/$04.00+0

Neutralizing Antibodies from the Sera of Human Immunodeficiency Virus Type 1-Infected Individuals Bind to Monomeric gp120 and Oligomeric gp140

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Received 1 July 1998/Accepted 9 September 1998

Antibodies that neutralize primary isolates of human immunodeficiency virus type 1 (HIV-1) appear during HIV-1 infection but are difficult to elicit by immunization with current vaccine products comprised of monomeric forms of HIV-1 envelope glycoprotein gp120. The limited neutralizing antibody response generated by gp120 vaccine products could be due to the absence or inaccessibility of the relevant epitopes. To determine whether neutralizing antibodies from HIV-1-infected patients bind to epitopes accessible on monomeric gp120 and/or oligomeric gp140 (ogp140), purified total immunoglobulin from the sera of two HIV-1-infected patients as well as pooled HIV immune globulin were selectively depleted of antibodies which bound to immobilized gp120 or ogp140. After passage of each immunoglobulin preparation through the respective columns, antibody titers against gp120 and ogp140 were specifically reduced at least 128-fold. The gp120- and gp140-depleted antibody fraction from each serum displayed reduced neutralization activity against three primary and two T-cell line-adapted (TCLA) HIV-1 isolates. Significant residual neutralizing activity, however, persisted in the depleted sera, indicating additional neutralizing antibody specificities. gp120- and ogp140-specific antibodies eluted from each column neutralized both primary and TCLA viruses. These data demonstrate the presence and accessibility of epitopes on both monomeric gp120 and ogp140 that are specific for antibodies that are capable of neutralizing primary isolates of HIV-1. Thus, the difficulties associated with eliciting neutralizing antibodies by using current monomeric gp120 subunit vaccines may be related less to improper protein structure and more to ineffective immunogen formulation and/or presentation.

Neutralizing antibodies (NAbs) are an important component of protective immunity against numerous viruses (7, 12, 23, 27–29, 64, 68), and most effective vaccines elicit pathogen-specific NAbs (reviewed in references 11 and 56). Since protection of humans against human immunodeficiency virus type 1 (HIV-1) infection has not been achieved, the role of NAbs in protective immunity against HIV-1 is not known. However, based on the experience with other viruses, it is reasonable to assume that NAbs play a role in protection against infection by HIV-1. Abs capable of neutralizing HIV-1 in vitro develop naturally, over several years, in HIV-infected patients (10, 31, 41, 44, 57, 60, 77, 78). However, immunization with monomeric forms of the HIV-1 envelope glycoprotein gp120 results in production of Abs which neutralize T-cell line-adapted (TCLA) viruses (4, 62, 65) but have only marginal activity against primary isolates of HIV-1 (41, 42, 66, 79). Possible explanations for this weak neutralizing activity against primary viral isolates, in contrast to the potent NAbs that can develop during natural infection, include the inaccessibility or absence of relevant epitopes on the immunogen. Monoclonal Abs (MAbs) which potently neutralize primary isolates can bind to gp120, but it has been suggested that the neutralizing capacity of these Abs correlates more closely with the efficiency of binding to epitopes exposed on the oligomeric form of gp120 (22, 45, 49, 59), as the oligomeric protein may more closely resemble the native structure of HIV-1 gp120/gp41 (21, 51, 61, 70). In support of these studies, recent work in our laboratory has shown that immunization of rabbits with oligomeric gp140 (ogp140) can elicit moderate levels of NAbs against some primary isolates (74).

Several experimental approaches have been used to identify the epitope specificity of NAbs from the sera of HIV-infected patients (3, 6, 37, 43, 53, 67, 75). In antibody depletion studies, Abs which bound to both linear and conformation-dependent epitopes of gp120 or to the V3 loop peptide of gp120 were found to have a role in the neutralization of TCLA viruses (3, 37, 43, 53, 67, 75). Work from our laboratory extended these results to show that V3-specific Abs had a marginal role in neutralizing primary viral isolates (75). In this study, we depleted sera of Abs which bind to monomeric gp120 or to ogp140 and evaluated their role in the neutralization of three primary isolates and two TCLA strains of HIV-1. We show that HIV-1 serum Abs directed to either monomeric gp120 or ogp140 can neutralize primary isolates of HIV-1. These data suggest that the epitopes important in mediating HIV-1 serum neutralization of primary isolates are present on subunit HIV-1 envelope (Env) glycoproteins and that further optimization of the presentation of these epitopes on vaccine products could improve their immunogenicity.

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MATERIALS AND METHODS

Cells and viruses. Peripheral blood mononuclear cells (PBMCs) were isolated by leukophoresis of blood from HIV-1- and hepatitis B virus-seronegative donors and then subjected to centrifugation over lymphocyte separation medium. PBMCs were stored in liquid nitrogen at 3×10^7 cells/ml in RPMI 1640 medium (Quality Biological Inc., Gaithersburg, Md.) containing 20% heat-inactivated fetal calf serum (FCS; PAA Laboratories Inc., Newport Beach, Calif.) and 10% dimethyl sulfoxide. For treatment with phytohemagglutinin (PHA; Difco Laboratories, Detroit, Mich.), cells were thawed and cultured for 24 h at 10^6 cells/ml in RPMI 1640 medium containing 15% FCS and 20 U of recombinant human interleukin-2 (IL-2; Boehringer, Mannheim, Germany) per ml (complete medium) in the presence of PHA at 1 μg/ml. Cells were washed free of the PHA-containing medium after 24 h and were incubated for an additional 48 to 72 h in complete medium. TCLA HIV-1_{IIIB} and HIV-1_{MN} and the primary isolate HIV-1_{US056} were obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health. HIV-1_{US1} and HIV-1_{CM237} are primary clade B isolates obtained from infected subjects from the National Naval Medical Center, Bethesda, Md., and Chiang Mai, Thailand, respectively. Virus titers were determined in PHA/IL-2-stimulated PBMCs.

Ab and protein reagents. Sera from patients US20 and US22, selected based on their broad and strong neutralization of primary HIV-1 isolates, were obtained with informed consent from clade B HIV-infected subjects. All HIV-1 sera and normal human sera (NHS; Sigma Chemical Co., St. Louis, Mo.) were heat inactivated at 56°C for 30 min prior to use. The purified polyclonal immunoglobulin (purified Ig) fraction was purified from sera US20 and US22 by using an EZ-SEP kit (Pharmacia Biotech, Piscataway, N.J.) prior to affinity column depletion and purification. To ensure that equivalent amounts of purified Ig and serum were used in subsequent binding and virus neutralization assays, the volume of purified Ig was adjusted to return the titers of Abs to gp120, ogp140, and p24 to the levels of unfractionated HIV-1 sera. Samples were concentrated in Centricon 30 spin concentrators (Amicon, Danvers, Mass.) with multiple spins at $5{,}000 \times g$ for 25 min. HIV-1 immune globulin (HIVIG; 50 mg/ml) is a preparation of purified polyclonal Ig derived from the plasma of multiple HIV-infected donors and was kindly provided by Chris Saban (NABI, Boca Raton, Fla.). The donors contributing to HIVIG were clinically asymptomatic and had CD4 lymphocyte counts greater than or equal to 400 cells/ml, high anti-p24 antibody titers, and undetectable p24 antigen (16). MAb T4 was kindly provided by Pat Earl (National Institutes of Health) (20). Monomeric $gp120_{451}$ and $ogp140_{451}$ were affinity purified from the culture medium of a cell line (6D5) chronically infected with HIV-1_{451} as described previously (35, 36, 76). The $ogp140_{451}$ preparation is comprised mostly of trimers/tetramers and dimers, with some monomers (76), and is a truncated version of full-length gp160, with the truncation occurring just prior to the transmembrane domain. Recombinant p24 was obtained from MicroGeneSys Inc. (Meriden, Conn.); $V3_{MN}$ peptide (YNKRKRIHIGPGRAFYTTKNIIGC), corresponding to the V3 region of gp120, and gp41 peptide $gp41_{582}$ (QARILAVERYLKDQQLLGIWGCSGKLIC), corresponding to the immunodominant domain of gp41 and contained within $ogp140_{451}$, were synthesized by Synthecell (Gaithersburg, Md.). Reduced, carboxymethylated $gp120_{MN}$ was generously provided by Genentech Inc. (South San Francisco, Calif.).

Column depletion. $gp120_{451}$ and $ogp140_{451}$ were coupled to separate CNBr-activated Sepharose 4B beads as described by the manufacturer (Pharmacia Biotech). Prior to incubation with purified Ig derived from sera of HIV-1-infected patients or with NHS, 0.4 ml of packed gp120 or ogp140-coupled Sepharose 4B beads was treated in a 2-ml polystyrene column (Pierce, Rockford, Ill.) with 0.4 ml of NHS to reduce nonspecific binding of Abs. The beads were then washed with 0.8 ml of phosphate-buffered saline (PBS) and incubated with 0.2 ml of purified Ig and 0.4 ml of PBS in a 1.5-ml tube for 4 h at room temperature on a rotating wheel. The mixture was transferred to a polystyrene column, and the eluate (depleted fraction) was collected. The beads in the column were washed twice with 0.4 ml of PBS, and each wash was added to the main eluate. The combined eluate was then reincubated with a fresh 0.4 ml of packed, coupled Sepharose beads for an additional 4 h at room temperature on a rotating wheel. This material was transferred to a new column, and the eluate was collected. These beads were washed three times with 0.4 ml of PBS, and the washes were added to the main eluate. The eluate combined with the three washes was considered the final depleted fraction. gp120- or ogp140-depleted fractions were concentrated in Centricon spin concentrators to a volume at which the enzyme-linked immunosorbent assay (ELISA) titer of anti-p24 Abs was equal to that of the undepleted Ig. In the case of depleted NHS, the sample was concentrated to the original total IgG concentration.

The gp120- or ogp140-coupled Sepharose beads with bound serum Abs were washed five times with 1 ml of PBS, followed by two washes with 1 ml of 500 mM NaCl to remove weakly or nonspecifically associated Abs. For US20 and US22, Ab was eluted with 2.5 ml of 100 mM Na_2CO_3, pH 11. For HIVIG, Ab was eluted with 100 mM Na_2CO_3 as described above, followed by addition of 1.8 ml of 100 mM H_3PO_4, pH 2. The efficiency of Ab recovery was improved twofold by this sequence of high- and low-pH elution. The high- and low-pH solutions containing eluted Abs were neutralized with 1 N HCl and 1 M $NaPO_4$, respectively. All data for affinity-purified Abs from HIVIG refer to Abs combined from the high- and low-pH elutions, except for the HIVIG data shown in Table 2, which are for Na_2CO_3-eluted Abs alone. Columns were prepared for reuse by serial washes with 100 mM Na_2CO_3 and 100 mM H_3PO_4 followed by extensive washing with PBS. Eluted Abs were concentrated to the original volume in Centricon 30 spin concentrators as described above. Where indicated, $V3_{MN}$ peptide was immobilized to Sepharose, and Ab depletion and affinity purification were done as described above and previously (76).

Ab binding levels in depleted and affinity-purified Ig samples. Unfractionated serum, purified Ig, and column-depleted and affinity-purified Ab fractions were assayed for amount of Ab reactive to specific HIV-1 peptides and to subunit HIV-1 Env glycoproteins, or for the amount of total Ig, by ELISA as described previously (1, 72, 74). Briefly, $gp120_{451}$, $ogp140_{451}$, p24, or synthetic peptides $V3_{MN}$ and $gp41_{582}$ (1 μg/ml) in PBS (pH 7.4, with 0.01% thimerosal) were coated overnight at 4°C onto Immulon 2 microtiter plates (Dynatech, Chantilly, Va.). Plates were washed twice with wash buffer (PBS with 0.1% Tween 20, pH 7.4) prior to incubation with twofold dilutions of Ab-containing samples diluted in serum diluent (wash buffer with 5% skim milk, pH 7.4) for 1 h at 37°C. Plates were washed three times with wash buffer and incubated with horseradish peroxidase-conjugated goat anti-human IgG (diluted 1:8,000 in serum diluent) (Kirkegaard & Perry, Gaithersburg, Md.). After a 1-h incubation at 37°C, plates were washed three times, after which substrate [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS); Kirkegaard & Perry] was added. The reaction was stopped with 0.5% sodium dodecyl sulfate after 30 min at 37°C. Alternatively, total serum IgG concentrations were determined by coating plates with unlabeled anti-human IgG and detecting captured human IgG by using horseradish peroxidase-conjugated goat anti-human IgG as described elsewhere (1). Concentrations were determined by using a human IgG standard (Sigma). Relative levels of Ab specific for native and denatured forms of HIV-1 gp120 were determined by surface plasmon resonance (SPR) as described previously (72–74).

Virus neutralization assays. Virus neutralization assays were performed with PHA/IL-2-stimulated PBMCs by methods similar to those previously described (40, 41). Isolates of HIV-1 (100 50% tissue culture infective doses) were preincubated, in quadruplicate wells, with 5 to 8 serial 2- to 10-fold dilutions of HIV-1 sera, purified Ig, column-depleted Ab, or affinity-purified Ab in 0.05-ml 96-well culture plates (PGC, Frederick, Md.) for 45 min at 37°C. Controls included virus preincubated with NHS and PBS. Dilutions were made in RPMI 1640 containing 15% FCS. HIV-1 sera, purified Ig, and gp120- or ogp140-depleted Ab, previously normalized by ELISA reactivity to p24 antigen as described above, were prepared at the same initial sample dilutions. The initial dilutions of eluted $gp120_{451}$- and $ogp140_{451}$-specific purified Abs were adjusted so that the ELISA reactivity of each sample to gp120 or ogp140 was equivalent to that of the undepleted purified Ig. Then 1.5×10^5 PHA-activated PBMCs in 0.05 ml were added to the Ab-virus mixture. After incubation for 18 to 24 h at 37°C, the infected cells were washed five times with 0.45 ml of RPMI 1640 containing 15% FCS to remove unabsorbed virus and residual antibody to p24 (39). Cells were resuspended in 0.25 ml of complete medium, and 0.22 ml was distributed into wells of a 96-well tissue culture plate (Costar, Cambridge, Mass.). Culture supernatants from infected cells were tested on day 4, 5, or 6, depending on virus growth kinetics (40), and viral growth was determined by measuring p24 antigen in the culture medium by ELISA (Coulter, Miami, Fla.). Virus neutralization was determined by measurement of the fraction of remaining infectious virus after exposure to Ab. This value was obtained by dividing the amount of p24 antigen produced at each dilution of HIV-1 Abs by the amount produced in the absence of HIV-specific Abs. Fifty percent, 90%, and 99% neutralization titers were determined by linear regression analysis.

RESULTS

Efficient depletion of monomeric gp120- and ogp140-binding Abs from purified Ig of HIV-infected patients. To determine the relative importance of Abs specific for monomeric gp120 and ogp140 in neutralization of primary and TCLA isolates of HIV-1, purified Ig from three sources (US20, US22, and HIVIG) was selectively depleted of Abs which bound to $gp120_{451}$ or $ogp140_{451}$. To test the efficiency of the affinity column depletion and purification procedures, the titers of Abs in the depleted and affinity-purified fractions reactive with $ogp140_{451}$, $gp120_{451}$, p24, and two peptides, $V3_{MN}$ and $gp41_{582}$, were determined by ELISA. Screening against p24 antigen was included as a control to test and correct for nonspecific loss of Ig during the depletion procedure, thus permitting direct comparison of Ab titers in the various fractions against $ogp140_{451}$, $gp120_{451}$, $V3_{MN}$, and $gp41_{582}$. Peptide $gp41_{582}$, present only on $ogp140_{451}$, was included as an additional control for the specificity of the $gp120_{451}$ column. One of the samples, HIVIG, was also depleted of $V3_{MN}$-binding Abs by using a $V3_{MN}$-Sepharose column to confirm results of a previous study (75) and to

TABLE 1. Efficient removal of HIV-1 envelope-binding Abs from purified Ig from HIV-infected patients

Sample[a]	ELISA endpoint antibody titer against:				
	$ogp140_{451}$	$gp120_{451}$	$V3_{MN}$	$gp41_{582}$	p24
US20					
Purified Ig	409,600	51,200	12,800	6,400	204,800
gp120-depleted fraction	204,800 (2^b)	400 (128)	3,200 (4)	6,400 (1)	204,800
ogp140-depleted fraction	1,600 (256)	400 (128)	1,600 (8)	<400 (>16)	204,800
gp120 Abs	25,600	12,800	400	<100	<100
ogp140 Abs	51,200	12,800	400	400	800
HIVIG					
Purified Ig	1,638,400	409,600	25,600	102,400	1,600,000
gp120-depleted fraction	819,200 (2)	200 (2,048)	3,200 (8)	102,400 (1)	1,600,000
ogp140-depleted fraction	200 (8,192)	400 (1,024)	6,400 (4)	12,800 (8)	1,600,000
$V3_{MN}$-depleted fraction	819,200 (2)	409,600 (1)	<100 (>256)	ND[c]	1,600,000
gp120 Abs	102,400	102,400	3,200	<800	1,600
ogp140 Abs	409,600	102,400	3,200	25,600	3,200

[a] Purified Ig, purified Ig from HIV-1 serum; gp120-, ogp140-, and $V3_{MN}$-depleted fractions, HIV-1 sera depleted over gp120, ogp140, and $V3_{MN}$ columns, respectively; gp120 Abs and ogp140 Abs, Abs purified from HIV-1 serum by using gp120 and ogp140 affinity columns, respectively. Values for depleted fractions were normalized to the unfractionated Ig by correcting for the amount of Ab to p24, and affinity-purified (eluted) Abs were concentrated to the original volume of the Ig applied to each column, as described in Materials and Methods.

[b] Fold reduction compared to Ig.

[c] ND, not determined.

compare directly the roles of V3- versus gp120- and ogp140-specific Abs in the neutralization of primary and TCLA HIV-1 isolates.

ELISA titers of column-depleted and affinity-purified fractions against various antigens are shown in Table 1 for US20 and HIVIG. The data for US22 were similar and are not shown. As mentioned previously, the volumes of the gp120- and ogp140-depleted samples were adjusted such that the Ab responses against the control antigen p24 were equivalent to those of the purified Ig samples, as shown in Table 1. This required an approximately twofold concentration, indicating some nonspecific loss of antibody during the depletion procedure. While the p24-binding Ab titers for purified Ig and depleted samples were comparable, the $gp120_{451}$- and/or $ogp140_{451}$-binding Ab titers were substantially lower, indicating a selective depletion of HIV-1 envelope-specific Abs. The $gp120_{451}$-binding Ab titer of each purified Ig preparation was reduced at least 128-fold (greater than 99% reduction) after passage through the Sepharose-$gp120_{451}$ column (gp120-depleted fractions [Table 1]). Similarly, the $ogp140_{451}$ binding Ab titer of each purified Ig preparation after passage through the Sepharose-$ogp140_{451}$ column was reduced at least 256-fold ($ogp140_{451}$-depleted fractions [Table 1]). Abs reactive with $gp120_{451}$ were efficiently removed after passage through either the $gp120_{451}$ or $ogp140_{451}$ columns, while the majority of Abs reactive with $ogp140_{451}$ were efficiently removed by immobilized $ogp140_{451}$ but not by $gp120_{451}$, indicating the presence of a substantial proportion of $ogp140_{451}$-specific Abs reactive with epitopes either within gp41 or within gp120 but unique to its oligomeric configuration.

To characterize more precisely the accessibility of specific epitopes on the column-immobilized gp120 and ogp140 used in these experiments, the extent of depletion of $V3_{MN}$- and $gp41_{582}$-specific Abs on each column was determined. The $gp120_{451}$-depleted fraction retained the full reactivity by ELISA against the gp41 peptide ($gp41_{582}$), while reactivity was diminished in the gp140-depleted fraction. Interestingly, while reactivity to $gp41_{582}$ was reduced >16-fold in US20, only an 8-fold reduction was obtained for HIVIG despite the 8,192-fold reduction in reactivity against the entire $ogp140_{451}$ protein. There was also only a modest four- to eightfold reduction in Ab titer to $V3_{MN}$ in both the gp120- and gp140-depleted material from each purified Ig preparation, in contrast to the more than 256-fold reduction in V3-specific Abs after HIVIG was passed through the Sepharose $V3_{MN}$ column. Thus, depletion of Abs to the whole $gp120_{451}$ or $ogp140_{451}$ proteins by using the gp120 or ogp140 affinity columns was more efficient than depletion of Abs against specific immunodominant epitopes. However, while some Abs specific for immunodominant linear epitopes were less efficiently depleted, total gp120- and ogp140-specific Abs were reduced >99%.

Affinity-purified gp120- and ogp140-specific Abs retain binding capacity. Abs which had bound column-immobilized $gp120_{451}$ or $ogp140_{451}$ were eluted and evaluated for efficiency of recovery and specificity of reactivity by ELISA against the panel of proteins and peptides used in the assays described above for the depleted fractions. As shown in Table 1, there was minimal nonspecific binding of Abs to each column, as demonstrated by the small amount of affinity-purified Abs reactive with p24 (representing <0.5% of the initial p24 reactivity). Elution of Abs from either column and concentration of each fraction to its original volume resulted in recovery of approximately 25% of the initial gp120- or ogp140-specific binding activity (compare data for gp120 and ogp140 Abs with data for purified Ig). Similar percentages of $gp120_{451}$ reactivity were recovered from both the $gp120_{451}$ and $ogp140_{451}$ columns for all three purified Ig preparations (data shown for only US20 and HIVIG in Table 1). In contrast, Abs recovered from the ogp140 column displayed greater reactivity to ogp140 than did Abs recovered from the gp120 column, probably because of the presence of Abs to gp41 in the ogp140-purified Abs. These data demonstrate that gp120- and ogp140-specific Abs were selectively enriched by binding to the affinity columns and that the eluted affinity-purified Abs retained binding capacity.

Affinity-purified gp120- and ogp140-specific Abs bind preferentially to native gp120. To confirm that Sepharose-bound $gp120_{451}$ and $ogp140_{451}$ were capable of binding Abs specific for conformational epitopes within gp120, the $gp120_{451}$- and $ogp140_{451}$-depleted and affinity-purified Abs from HIVIG were analyzed by SPR for binding to conformationally intact gp120 and denatured (reduced, carboxymethylated) gp120. HIVIG bound preferentially to native gp120, with a native/

TABLE 2. gp120 and ogp140 columns selectively deplete and affinity purify Abs to conformational epitopes of gp120

Sample[a]	Ab binding to indicated antigen by SPR (RU)			Ratio, native gp120/ denatured gp120
	$ogp140_{451}$	Native $gp120_{MN}$	Denatured $gp120_{MN}$	
Purified Ig	3,499	3,545	492	7.2
$gp120_{451}$-depleted fraction	2,331	323	217	1.5
$ogp140_{451}$-depleted fraction	143	372	256	1.5
$V3_{MN}$-depleted fraction	2,645	2,569	53	48.3
$gp120_{451}$ Abs	1,324	3,523	571	6.2
$ogp140_{451}$ Abs	3,671	4,088	576	7.1
$V3_{MN}$ Abs	81	283	244	1.2

[a] All derived from HIVIG. For details of preparation, see Table 1, footnote *a*; the dilutions of affinity-purified (eluted) Abs were adjusted to yield gp120 or ogp140 binding levels equivalent to those of polyclonal Ig as described in Materials and Methods.

denatured gp120 binding ratio of 7.2 (Table 2), consistent with previous results for sera from HIV-1 infected individuals (46, 72). Both $gp120_{451}$- and $ogp140_{451}$-depleted fractions were preferentially depleted of Abs to native gp120. In both cases, there was a reduction in the native/denatured gp120 binding ratio from 7.2 (found in the unfractionated HIVIG) to 1.5 in the depleted samples. This was calculated by dividing antibody binding (in RU) to native, monomeric gp120 (3,542 RU) by binding to denatured gp120 (492 RU). In contrast, Abs to linear epitopes were selectively removed when HIVIG was depleted by using the $V3_{MN}$-coupled Sepharose beads. The native/denatured gp120 binding ratio rose to 48.3, consistent with the relative accessibility of the V3 region on native and denatured gp120. These data also indicate that the majority of denatured gp120-specific Abs within HIVIG are specific for V3. The affinity-purified $gp120_{451}$ and $ogp140_{451}$ Abs had reactivities to native and denatured gp120 similar to those of unfractionated HIVIG, with ratios of 6.2 and 7.1, respectively. These results demonstrate that both immobilized $gp120_{451}$ and $ogp140_{451}$ were able to bind Abs to conformational epitopes, suggesting that the tertiary structure of gp120 was retained in the column matrix. In addition, the presence of oligomeric gp140-specific epitopes within the immobilized gp140 column, but not the gp120 column, was demonstrated by using an oligomer-specific MAb (T4 [20]) that bound only to the former (data not shown). In summary, both $gp120_{451}$ and $ogp140_{451}$ columns depleted HIV-1 sera preferentially of Abs specific for conformational gp120 epitopes, and affinity-purified Abs from both columns preferentially bound native gp120.

Purified Ig from HIV-1 sera retains neutralizing activity against primary and TCLA HIV-1. To confirm that the viral inhibitory activities of the sera from HIV-infected patients US20 and US22 were Ig mediated and not due to other factors such as chemokines (13), the viral neutralization titers of purified Ig from these two sera against three primary isolates and two TCLA viruses were compared to the viral neutralization titers of the respective sera. Samples from the third source of purified Ig, HIVIG, were assayed similarly, but the original serum pool was not available for comparison. Of the three purified Ig preparations, US20 had the lowest titer of Abs to $gp120_{451}$ and $ogp140_{451}$, as measured by ELISA, yet demonstrated the strongest neutralization of the three HIV-1 primary isolates US1, CM237, and 056 (Table 3). The serum and purified Ig of US20 had approximately equal neutralization titers (ID_{50} and ID_{90}) against these viruses. US22 serum and purified Ig also had similar ID_{50} values against the primary isolates, although the ID_{90} of the purified Ig was two- to threefold less than that of the serum (Table 3). HIVIG had a high neutralization titer against $HIV\text{-}1_{CM237}$ but relatively low neutralization titers against $HIV\text{-}1_{US1}$ and $HIV\text{-}1_{056}$. All three purified Ig preparations had comparably strong neutralization activity against TCLA $HIV\text{-}1_{MN}$. Their neutralization titers against $HIV\text{-}1_{IIIB}$ were lower and more comparable to those observed against the primary isolates. These data demonstrate that the purified Ig from US20 and US22 sera retained most of the ability of the sera to neutralize primary and TCLA HIV-1. Neutralizing activity for these sera did not correlate with the total amount of Abs to monomeric $gp120_{451}$ or $ogp140_{451}$. This finding is consistent with other studies showing no correlation between HIV-1 Env-specific MAb binding titers to gp120 and neutralization (22, 45, 50, 59).

Depletion of gp120- and ogp140-specific Abs from HIV-1 sera diminishes neutralizing activity against TCLA and primary HIV-1. To determine the importance of gp120- and ogp140-specific Abs from individual sera in the neutralization of HIV-1, $gp120_{451}$- and $ogp140_{451}$-depleted fractions and affinity-purified Abs from US20 and US22 were evaluated for neutralizing capacity against TCLA and primary HIV-1 isolates. After standardization of the depleted fractions to the purified Ig by the amount of Abs to p24, the gp120- and ogp140-depleted fractions from US20 and US22 had reduced neutralizing activity against all viruses evaluated (Fig. 1A; Table 4). The level of reduction in neutralization was similar for the $gp120_{451}$- and $ogp140_{451}$-depleted fractions. The increase

TABLE 3. Ab binding and viral neutralization titers of sera and purified Ig from HIV-1-infected patients

Sample[a]	ELISA titer of Abs reactive with:		50%/90% viral neutralization titer against:				
			Primary isolate			TCLA isolate	
	$gp120_{451}$	$ogp140_{451}$	US1	CM237	056	IIIB	MN
Patient US20							
Serum	51,200	409,600	240/138	302/161	187/111	1,967/734	3,409/973
Purified-Ig	51,200	409,600	204/121	298/152	209/121	1,191/452	2,527/954
Patient US22							
Serum	409,600	3,276,800	70/15	67/29	38/11	120/40	4,502/1,943
Purified-Ig	409,600	3,276,800	53/<5	56/10	45/<5	114/21	3,783/1,658
HIVIG	409,600	1,638,400	22/13	281/36	15/5	97/65	3,692/907

[a] The Ig fractions of US20 and US22 were normalized to the respective sera for Ab reactivity to $gp120_{451}$, $ogp140_{451}$, and p24 as described in Materials and Methods.

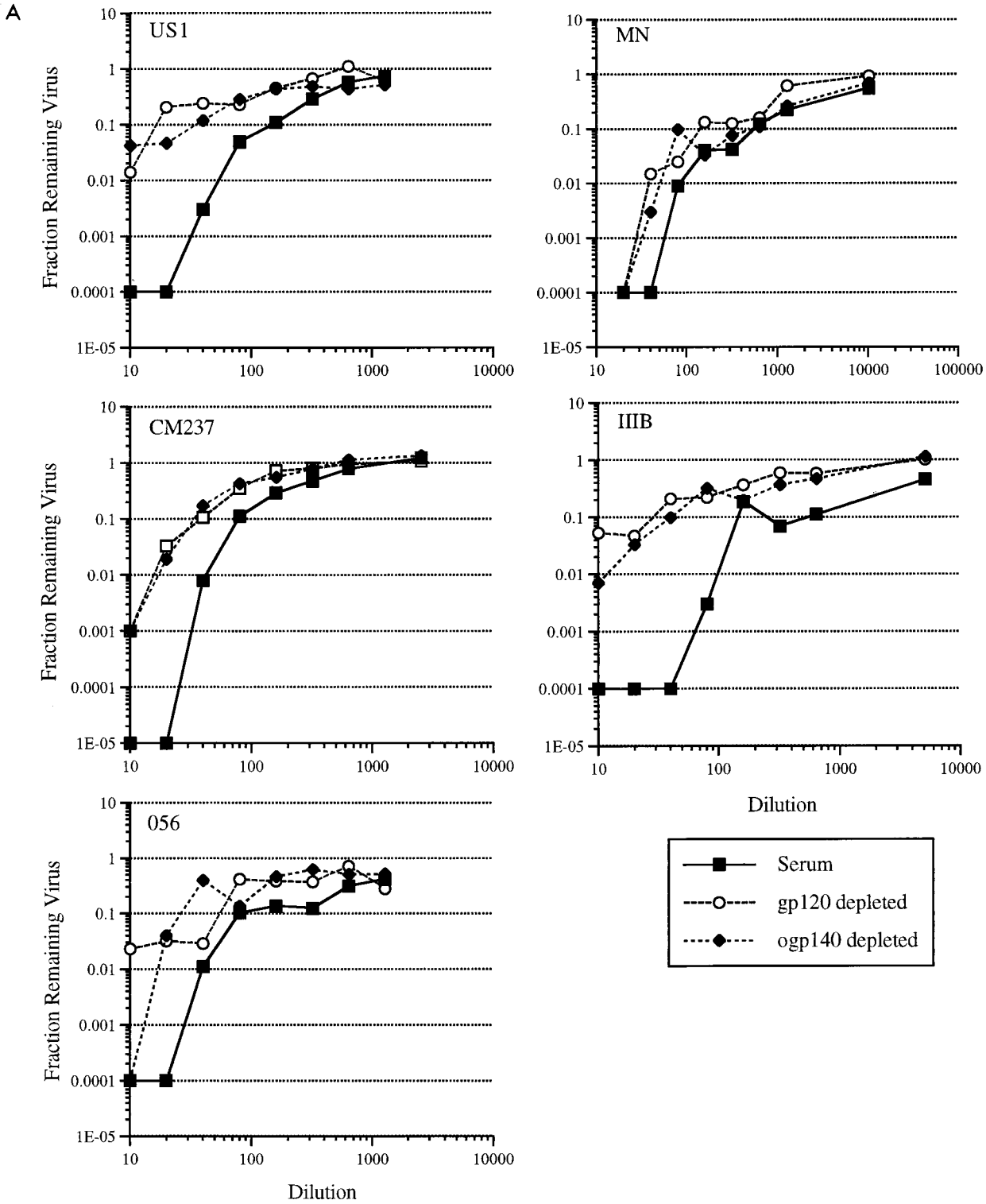

FIG. 1. Preparation of purified Ig and depleted samples, design of viral neutralization assays, and evaluation of p24 antigen were as described in Materials and Methods. (A) Results for gp120- and ogp140-depleted material from US20; (B) results for HIVIG. Each point represents the average from triplicate wells from one experiment of four that gave similar results. Depleted fractions were standardized to the purified Ig and serum by equating reactivity to p24 such that equivalent amounts of each were added. Diluted samples were evaluated by ELISA for titers of Abs to p24 after the neutralization assay to confirm that equivalent amounts were added. The amount of viral growth in comparable dilutions of NHS was used as the standard for 100% virus growth. Purified Ig and depleted fractions were assessed for neutralizing capacity against primary (US1, CM237, and 056) and TCLA (MN and IIIB) HIV-1 isolates. gp120-depleted and ogp140-depleted fractions correspond to HIV-1 serum US20 or HIVIG depleted over the gp120 or ogp140 affinity columns, respectively.

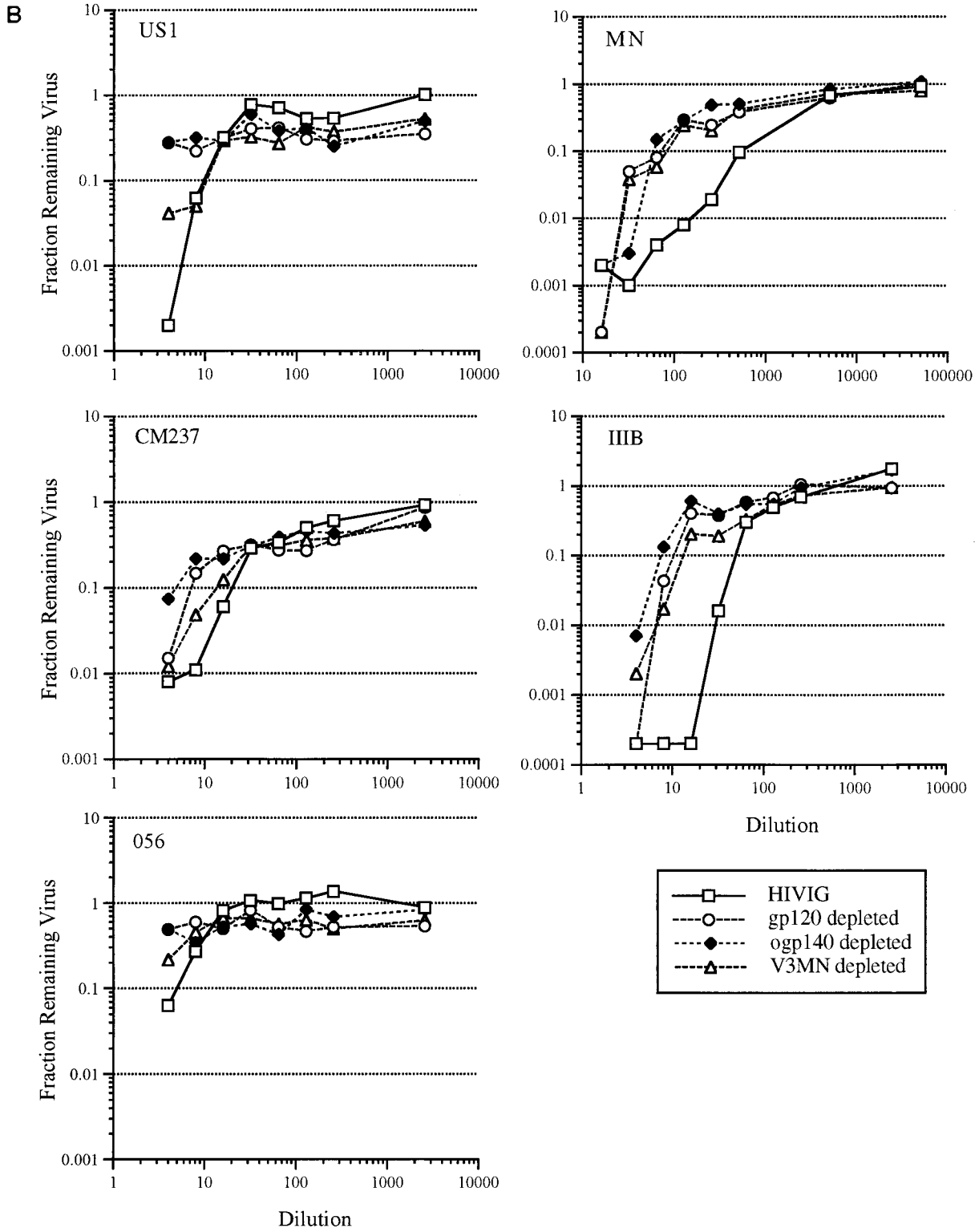

FIG. 1—*Continued.*

in virus growth in the presence of the gp120- and gp140-depleted fractions was most striking against HIV-1_{IIIB}, with a 2- to 3-$\log_{10}$ difference between the depleted and unfractionated material (Fig. 1A). In the presence of lower dilutions of depleted US20 fractions, a greater than 100-fold increase in virus growth of HIV-1_{US1}, HIV-1_{CM237}, and HIV-1_{056} primary isolates was obtained (Fig. 1A). The corresponding ID_{90} and ID_{99} (neutralizing titers) of the depleted US20 material against the primary isolates of HIV-1 were reduced 2- to 10-fold (Table 4). The more weakly neutralizing US22 serum had a 2- to

TABLE 4. Viral neutralization titers of depleted and affinity-purified US20, US22, and HIVIG

Serum	Sample[b]	Neutralization titer[a]														
		Primary isolate									TLCA isolate					
		US1			CM237			056			IIIB			MN		
		50%	90%	99%	50%	90%	99%	50%	90%	99%	50%	90%	99%	50%	90%	99%
US20	Expt A															
	Serum	240	138	62	302	161	66	187	111	52	1,967	734	179	3,409	973	162
	Purified Ig	204	121	58	298	152	58	209	121	55	1,191	452	113	2,527	954	237
	gp120 depleted	132	27	<10	100	51	20	188	48	<10	218	28	<10	362	181	68
	ogp140 depleted	178	32	<10	102	50	18	106	57	23	89	39	12	475	230	82
	Expt B															
	Purified Ig	256	97	24	280	158	69	209	117	51	2,633	179	<20	2,399	904	224
	gp120 Abs	335	149	47	224	73	<20	229	33	<20	ND[c]	ND	ND	5,217	1,073	112
	ogp140 Abs	405	239	113	398	180	58	242	132	56	2,907	873	156	3,497	1,240	281
US22	Expt A															
	Serum	70	15	<5	67	29	9	38	11	<5	120	40	8	4,502	1,943	584
	Purified Ig	53	<5	<5	56	10	<5	45	<5	<5	114	21	<5	3,783	1,658	509
	gp120 depleted	<5	<5	<5	23	<5	<5	9	<5	<5	<5	<5	<5	1,401	460	<100
	ogp140 depleted	<5	<5	<5	12	<5	<5	<5	<5	<5	<5	<5	<5	4,799	<100	<100
	Expt B															
	Purified Ig	36	<10	<10	44	11	<10	32	<10	<10	264	<25	<25	3,783	1,658	509
	gp120 Abs	34	<10	<10	18	<10	<10	67	<10	<10	156	<25	<25	9,320	3,370	786
	ogp140 Abs	32	<10	<10	21	<10	<10	35	<10	<10	153	<25	<25	13,963	7,401	2,985
HIVIG	Expt A															
	Purified Ig	22	13	6	281	36	4	15	5	<4	97	65	37	3,692	907	122
	gp120 depleted	<4	<4	<4	62	11	<4	<4	<4	<4	24	16	9	140	69	25
	ogp140 depleted	<4	<4	<4	83	4	<4	<4	<4	<4	23	10	<4	192	95	35
	$V3_{MN}$ depleted	40	10	<4	165	21	<4	11	<4	<4	21	13	7	129	78	38
	Expt B															
	Purified Ig	22	13	6	281	36	4	15	5	<4	97	65	37	3,692	907	122
	gp120 Abs	43	4	<4	84	<4	<4	ND	ND	ND	ND	ND	ND	2,889	639	74
	ogp140 Abs	65	<4	<4	86	6	<4	ND	ND	ND	ND	ND	ND	2,227	757	161

[a] Neutralization titers for unfractionated, depleted, and affinity-purified Ig were determined by linear regression analysis of data such as those shown in Fig. 1 and 2, using an Excel computer program.

[b] Data for experiments A and B are from separate neutralization assays, evaluating depleted and affinity-purified material, respectively. The same polyclonal Ig samples were included for both experiments.

[c] ND, not done.

10-fold reduction in ID_{50} against these viruses (Table 4). Despite the reduction of neutralizing capacity against primary and TCLA HIV-1 isolates in both the gp120- and gp140-depleted US20 fractions, the remaining Abs in these depleted fractions retained a substantial (i.e., greater than 90% neutralization at the higher Ig concentration) level of neutralizing activity (Fig. 1A; Table 4).

Previous data showed that V3-specific Abs were important in mediating neutralization of TCLA (37, 53, 75) but not primary (75) HIV-1 isolates. To confirm these previous studies using HIVIG and to provide a basis for comparison with gp120- and ogp140-depleted samples, HIVIG was passed through a $V3_{MN}$-coupled Sepharose column, resulting in a >256-fold reduction in $V3_{MN}$-specific Abs (Table 1). In agreement with previous findings, neutralization of TCLA HIV-1_{MN} by $V3_{MN}$-depleted HIVIG was substantially reduced (≥20-fold against MN), with minimal corresponding reduction in neutralizing capacity against the primary isolates US1, CM237, and 056 (Table 4; Fig. 1B). In contrast, both gp120- and ogp140-depleted HIVIG had reduced primary isolate neutralization capacity. For example, while $V3_{MN}$-specific depletion failed to achieve a twofold reduction in ID_{50} and ID_{90} against primary HIV-1 isolates, gp120- and ogp140-specific depletion reduced the ID_{50} and ID_{90} approximately fourfold or more. The reduction in neutralizing titer of gp120- and ogp140-depleted HIVIG against TCLA isolate IIIB was comparable to the reduction against the primary HIV-1 isolates (Table 4), while a greater reduction in MN neutralizing titer was obtained. As obtained previously with US20, despite the reduction in primary isolate neutralizing capacity, in the case of CM237, significant neutralizing Abs remained in the gp120- and ogp140-depleted HIVIG.

To confirm that the procedures for purified Ig purification and Ab depletion, elution, and concentration did not introduce factors which nonspecifically inhibited viral growth, each experiment included negative controls in which the purified Ig from NHS was processed on the gp120 and ogp140 columns identically to the HIV-1 samples. Neither the purified Ig, the mock-depleted gp120 and ogp140 fractions, nor the mock-purified Abs from NHS had detectable neutralization against the viruses used in the study (data not shown). As an additional control, all HIVIG samples evaluated above were subjected to an identical experiment substituting a clade E HIV-1 isolate (9461 [74]), against which the clade B HIVIG had marginal neutralizing activity. None of these samples inhibited or enhanced viral growth of the clade E isolate.

gp120 and ogp140 affinity-purified antibodies neutralize both TCLA and primary HIV-1 isolates. US20, US22, and HIVIG affinity-purified Abs from the gp120 and ogp140 columns were tested for neutralizing activity against both TCLA and primary HIV-1 isolates. Prior to evaluation for neutralization activity, the concentrations of gp120- and ogp140-affinity

purified Abs in the Ab fractions were adjusted so that the ELISA titer against gp120 or ogp140 was equivalent to that of unfractionated purified Ig. Abs recovered from both gp120 and ogp140 columns from US20 had substantial neutralization activity against the two TCLA and three primary isolates of HIV-1 (Fig. 2A; Table 4). This activity was quantitatively similar to that of the unfractionated purified Ig. Furthermore, to compare the potencies of the gp120 and ogp140 Abs, the concentration of IgG in the initial dilution of each sample from US20 was determined and found to be as follows: serum, 645.5 μg/ml; purified Ig, 648.7 μg/ml; gp120 Abs, 10.1 μg/ml; and ogp140 Abs, 29.7 μg/ml. Based on IgG concentration, the 99% inhibitory concentrations against HIV-1_{US1} of gp120- and ogp140-specific Abs were similar at 4 and 5 μg/ml, respectively (data not shown), suggesting similar neutralizing potencies of the gp120 and ogp140 affinity-purified Abs. gp120- and ogp140-specific Abs from US22 and HIVIG also neutralized both TCLA and primary HIV-1 isolates, but to a more limited degree than US20 (data shown for HIVIG in Fig. 2B and Table 4). Thus, both gp120- and ogp140-specific, affinity-purified Abs from HIV-infected patients had potent neutralizing activity (90 to 99.9% at the lowest dilutions studied) against primary isolates and TCLA HIV-1.

DISCUSSION

We have demonstrated that HIV-1 serum Abs which neutralize primary HIV-1 isolates bind to both soluble monomeric $gp120_{451}$ and $ogp140_{451}$. Monomeric gp120 and ogp140 coupled to Sepharose beads efficiently removed >99% of serum Abs specific for the homologous protein. Elution of bound Abs from both gp120 and ogp140 columns yielded gp120- and ogp140-specific Abs which were normalized to original anti-Env reactivity and evaluated for HIV-1 neutralizing activity. The affinity-purified antibodies specific for gp120 were directed predominantly to epitopes exposed on native gp120. Less efficient depletion by gp120 or ogp140 was obtained against specific immunodominant epitopes such as V3 ($V3_{MN}$) and the immunodominant domain in gp41 ($gp41_{582}$), suggesting either reduced accessibility of these epitopes on immobilized gp120 or ogp140, differences in amino acid sequence of the epitope in the protein used to deplete (i.e., 451) and the peptide used for the antibody binding assay (MN or LAI), or insufficient concentration of the epitope within the protein immobilized on the column. For example, column immobilized peptide $V3_{MN}$ was capable of complete depletion of HIV-1 serum V3 antibody, but the concentration of the V3 region in the V3 affinity column material was approximately 100-fold higher than the concentration of V3 in the gp120 or ogp140 affinity columns. Therefore, while the total gp120- and ogp140-binding Ab titers were reduced 100-fold, some epitope-specific Ab populations may have been less efficiently depleted.

Removal of more than 99% of gp120- or ogp140-binding Abs from the sera of HIV-infected patients resulted in a significant decrease in neutralization titers against three primary and two TCLA isolates of HIV-1. This was in contrast to V3-depletion of HIV-1 sera in this study as well as in a previous study (74), where removal of V3 antibodies reduced neutralization against TCLA but not primary HIV-1 isolates. These data suggest the presence of antibodies with specificities outside of linear V3 epitopes and present on both gp120 and ogp140 with neutralizing activity against multiple primary HIV-1 isolates. In addition, affinity-purified $gp120_{451}$- and $ogp140_{451}$-specific Abs neutralized the infectivities of both TCLA and primary HIV-1 isolates. The gp120-specific Abs purified from both the $gp120_{451}$ and $ogp140_{451}$ affinity columns were predominantly directed to epitopes present on native but not denatured gp120, consistent with observations that HIV-1 serum gp120-specific Abs are directed predominantly to native gp120 (46, 72) and that many broadly neutralizing MAbs are specific for discontinuous epitopes within gp120 (9, 26, 52, 71). MAbs specific for HIV-1 gp41 which potently neutralize HIV-1 have been identified (14, 15, 47); however, the comparable HIV-1-neutralizing activities of gp120 and ogp140 affinity-purified Abs from HIVIG, US20, and US22 suggest that a significant portion of the primary HIV-1 isolate-neutralizing activity of polyclonal serum Ig is present in Abs with gp120 epitope specificities. For example, Abs from US20 recovered from the gp120 and ogp140 columns had comparable neutralizing potencies against HIV-1_{US1} as well as HIV-1_{CM237} and HIV-1_{056}. Therefore, the presence of gp41-specific antibodies in the ogp140 affinity-purified fraction did not contribute to enhanced neutralizing activity. It remains to be determined whether fine epitope specificities of the gp120-specific Abs from the gp120 and ogp140 affinity-purified sera are similar or whether the two fractions have distinct antibody populations with comparable neutralizing activities.

The oligomeric protein used in this study is comprised of a truncated gp160 (gp140) which is secreted from a chronically infected cell line derived from HUT78 cells. Although it naturally assembles and is secreted in culture media as monomers, dimers, and trimers/tetramers (76), the extent of resemblance, after being immobilized onto Sepharose, to native gp120/gp41 expressed on the surface of virions and infected cells is not clearly defined. This may help explain why despite extensive depletion of Abs to conformational epitopes on ogp140 (a 256-fold reduction in titer against $ogp140_{451}$), substantial neutralizing activity (greater than 90% of total neutralizing activity of US20 Ig) remained in the depleted fraction. NAbs specific for the C terminus of gp41 (truncated in ogp140) or to conformational epitopes of gp120/gp41 requiring proper quaternary structure dependent on either membrane expression or the presence of an entire intact gp41 may not have been absorbed from the sera analyzed. The depletion studies, by selectively removing gp120 conformational antibody, demonstrated the presence of some properly folded gp120 within the gp120- and gp140-coupled matrices. Binding of MAb T4, which maps to an oligomer-specific epitope within gp41 (19, 20), indicated the presence of some oligomeric gp140 after coupling to the column.

Another explanation may be the presence of NAbs type specific for the various primary isolates evaluated which were not efficiently depleted with the 451 strain of gp120 and ogp140 used for this study. These data indicate a significant proportion (up to 50%) of type-specific primary isolate NAb. Additionally, critical gp160 epitopes may be absent on the column-immobilized gp120 or ogp140, possibly secondary to immobilizing the proteins onto the Sepharose beads. For example, gp120 has been shown to undergo conformational changes after binding CD4 that result in enhanced accessibility or exposure of previously cryptic epitopes (17, 18, 24, 58, 69). Recent HIV-1 envelope structural data have also identified conserved regions of gp120 involved with interactions with CD4 and/or coreceptors which are hidden on the native gp120 protein by glycosylation and the hypervariable loop domains (38, 55, 80). Abs directed to these regions may be induced during natural infection but would not be expected to be efficiently depleted by using soluble gp120 and ogp140. Finally, NAbs directed against nonenvelope (8, 32, 48, 63) or nonvirus-encoded proteins (5, 25, 34, 54) which also would not have been depleted by using the HIV-1 Env-specific reagents have been identified. It should be noted that the inability to remove completely serum neu-

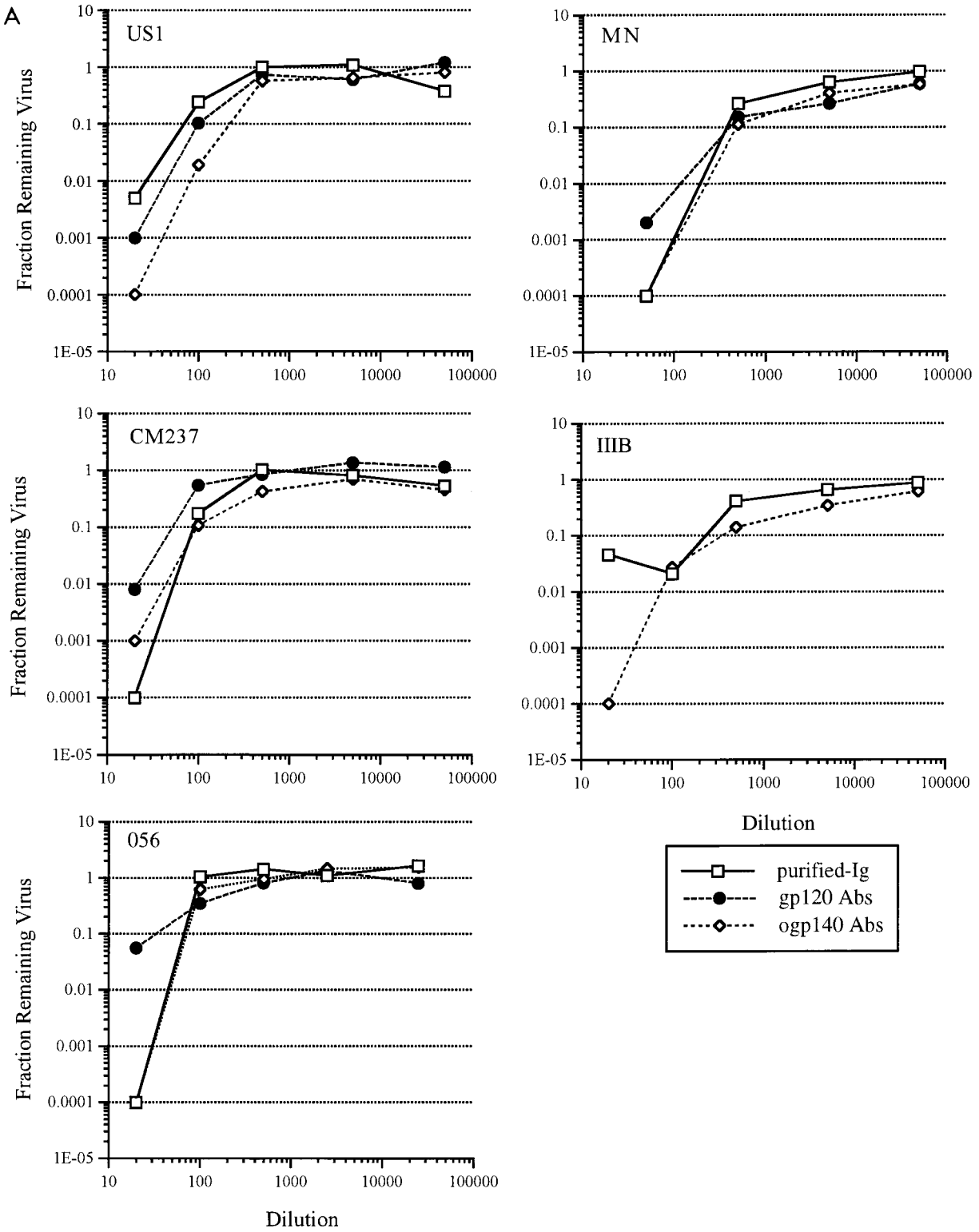

FIG. 2. Affinity-purified antibodies from the $gp120_{451}$ and $ogp140_{451}$ affinity columns for US20 (A) and from HIVIG (B) were compared with the respective unfractionated Ig for neutralization activity against primary (US1, CM237, and 056) and TCLA (MN and IIIB) HIV-1 isolates. Abs affinity purified from the monomeric gp120 column (gp120 Abs) or ogp140 column (ogp140 Abs) were standardized to the unfractionated serum and/or purified Ig by adding equivalent amounts of reactivity to gp120 or ogp140, respectively. Dilutions were evaluated by ELISA for titers to gp120 and/or ogp140 after the assay to confirm that equivalent amounts of reactivity had been added.

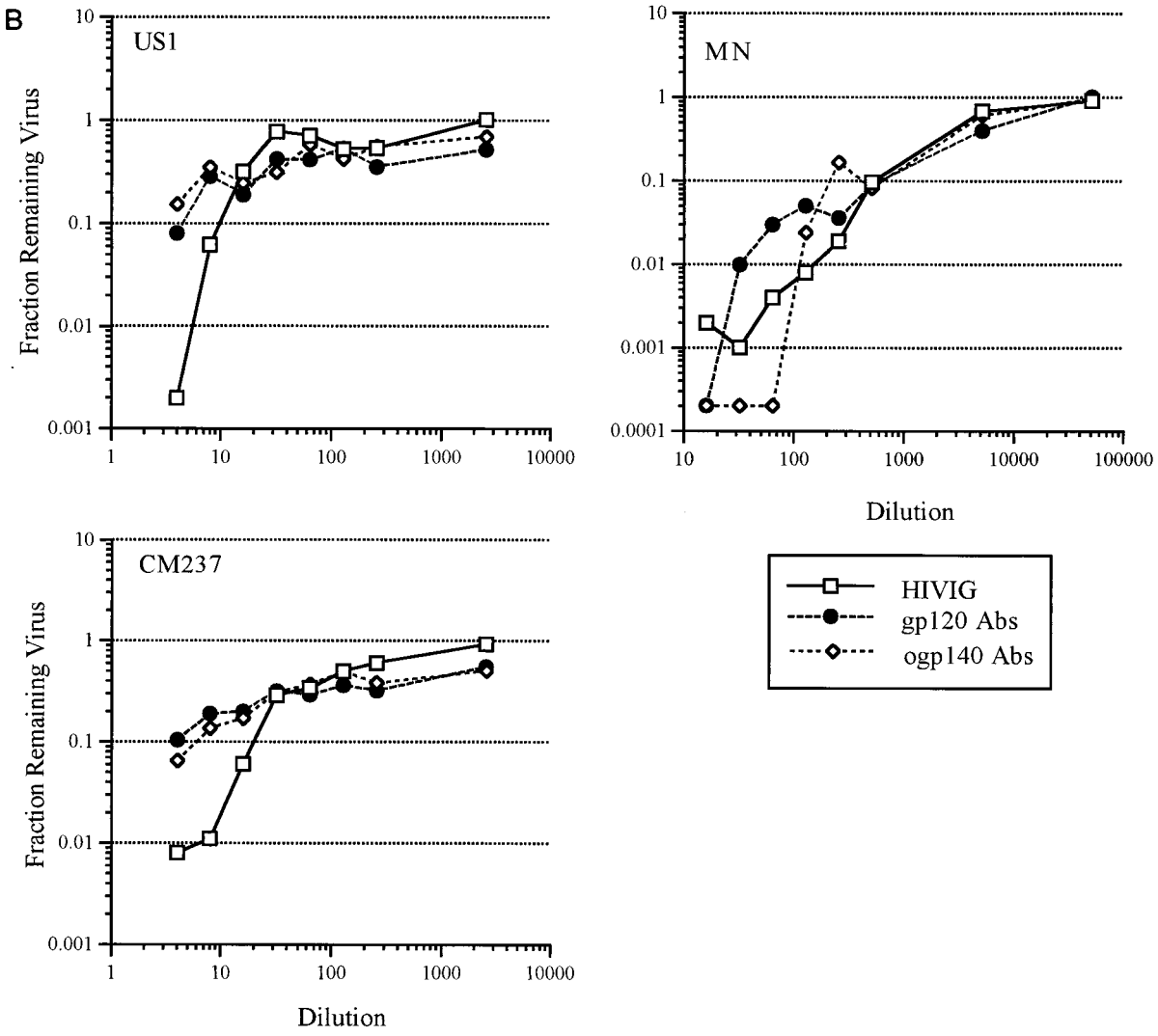

FIG. 2—*Continued.*

tralizing activity by binding to immobilized gp120, even when evaluated against the homologous TCLA virus (43, 67), has been observed previously.

The presence of epitopes accessible on both monomeric gp120 and ogp140 (antigenicity) capable of depleting HIV-1 serum neutralizing activity against primary HIV-1 isolates as well as affinity purifying broadly neutralizing Ab activity is inconsistent with their role as immunogens. Monomeric forms of gp120 have proven effective in eliciting NAb against TCLA HIV-1 isolates but with limited neutralizing activity against primary HIV-1 isolates (2, 30, 33, 41, 42, 62, 78). ogp140 has been shown to elicit Abs capable of neutralizing some primary HIV-1 isolates, but these responses have been restricted to date to those which are particularly susceptible to Ab-mediated neutralization (74). Broadly neutralizing Ab responses against primary isolates as observed with the gp120 and ogp140 affinity-purified sera have not been achieved in vaccine studies. Therefore, there appears to be a significant dislinkage between antigenicity and immunogenicity of soluble HIV-1 subunit vaccines. This may be related to structural instability of critical conformational epitopes within gp120 as expressed in gp120 or ogp140 and a more stringent requirement for eliciting an immune response. Potent NAbs may be capable of binding with lower affinity to these soluble HIV-1 envelope preparations but not effectively elicited by using these same proteins. Dose, route, timing of immunizations, and adjuvant formulations have been shown to qualitatively alter the quality of the Ab immune response in rabbits after immunization with ogp140 (74). This finding raises the possibility that further stabilization of immunogen, by oligomerization or adjuvant selection, may preserve neutralizing epitopes. Alternatively, in natural infection, potent NAbs against heterologous primary viral isolates develop over several years, and therefore more optimal maturation of the immune response may be required to elicit NAbs by immunization.

Our laboratory is continuing to identify epitopes on gp120/gp41 which are recognized by NAbs, with the goal of applying this knowledge toward design of an immunogenic vaccine. Comparison of the epitope specificities of gp120- and ogp140-specific Abs from a potently neutralizing HIV-1 serum with similarly purified Abs from vaccinee sera should help identify gaps in the latter. In addition, we are determining whether the remaining neutralizing activity in the $gp120_{451}$- and $ogp140_{451}$-depleted fractions, similar to what was seen in other studies

(43, 67), is related to heterologous NAbs which did not bind to $gp120_{451}$ and $ogp140_{451}$ or to Abs with epitope specificities not optimally presented by gp120 or ogp140. Finally, extensions of these type of studies to non-clade B HIV-1 isolates will be important to determine the relative contribution of various HIV-1 Env-specific antibodies to HIV-1 isolates circulating in areas where vaccine efficacy trials may be performed.

ACKNOWLEDGMENTS

We thank patients and staff of the Infectious Diseases Clinic at the National Naval Medical Center, Bethesda, Md. for sera used in these studies. We also thank I. Kalisz for technical assistance, C. Drew for graphic support, C. Sapan for HIVIG, P. Earl for MAbs, L. Loomis-Price for helpful discussions, and P. Gomatos for review of the manuscript.

This work was supported in part by cooperative agreement DAMD17-93-V-3004, between the U.S. Army Medical Research and Material Command and the Henry M. Jackson Foundation for the Advancement of Military Medicine.

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