JOURNAL OF VIROLOGY, Oct. 1997, p. 7198–7206 Vol. 71, No. 10
0022-538X/97/$04.00+0

Potent and Synergistic Neutralization of Human Immunodeficiency Virus (HIV) Type 1 Primary Isolates by Hyperimmune Anti-HIV Immunoglobulin Combined with Monoclonal Antibodies 2F5 and 2G12

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Received 5 March 1997/Accepted 19 June 1997

Three antibody reagents that neutralize primary human immunodeficiency virus type 1 (HIV-1) isolates were tested for magnitude and breadth of neutralization when used alone or in double or triple combinations. Hyperimmune anti-HIV immunoglobulin (HIVIG) is derived from the plasma of HIV-1-infected donors, and monoclonal antibodies (MAbs) 2F5 and 2G12 bind to distinct regions of the HIV-1 envelope glycoprotein. The antibodies were initially tested against a panel of 15 clade B HIV-1 isolates, using a single concentration that is achievable in vivo (HIVIG, 2,500 μg/ml; MAbs, 25 μg/ml). Individual antibody reagents neutralized many of the viruses tested, but antibody potency varied substantially among the viruses. The virus neutralization produced by double combinations of HIVIG plus 2F5 or 2G12, the two MAbs together, or the triple combination of HIVIG, 2F5, and 2G12 was generally equal to or greater than that predicted by the effect of individual antibodies. Overall, the triple combination displayed the greatest magnitude and breadth of neutralization. Synergistic neutralization was evaluated by analyzing data from dose-response curves of each individual antibody reagent compared to the triple combination and was demonstrated against each of four viruses tested. Therefore, combinations of polyclonal and monoclonal anti-HIV antibodies can produce additive or synergistic neutralization of primary HIV-1 isolates. Passive immunotherapy for treatment or prophylaxis of HIV-1 should consider mixtures of potent neutralizing antibody reagents to expand the magnitude and breadth of virus neutralization.

Passive administration of specific antibody can protect against disease caused by numerous viruses, including poliovirus, measles virus, rubella virus, mumps virus, varicella-zoster virus, rabies virus, hepatitis A and hepatitis B viruses, respiratory syncytial virus, and cytomegalovirus (3, 4, 28, 30, 32, 36, 41, 42, 57, 59, 61). Passive antibody may also be of some therapeutic benefit for ongoing viral infections due to respiratory syncytial virus, cytomegalovirus, parvovirus B19, and vaccinia virus (12, 23, 26, 30). Thus, numerous investigators have considered the possibility that passive immunity plays a role in the treatment and prevention of human immunodeficiency virus (HIV) infection. Initial treatment studies used HIV-immune plasma or immunoglobulin products derived from HIV-infected subjects (60). In some studies, administration of passive antibody to HIV-infected patients was associated with reduced p24 antigen (Ag) levels and/or increased $CD4^+$ T-lymphocyte counts (34, 39, 45, 54, 73); a few reports also suggested a decreased incidence of opportunistic infections or a trend toward improved overall survival (34, 35, 45, 54, 73). Preventive studies utilizing anti-HIV immunoglobulin (HIVIG) preparations and an anti-V3 monoclonal antibody (MAb) protected chimpanzees from infection with HIV-IIIB, a T-cell-line-adapted strain (22, 24, 56). More recently, Conley et al. administered human MAb 2F5 to two chimpanzees, followed by intravenous challenge with a primary HIV-1 isolate (15). While neither animal was protected from infection, both demonstrated substantial delays in plasma viremia, and one animal had a reduced viral load compared to control animals through 1 year of follow-up. Finally, the ability of passive antibody to interrupt maternal-infant transmission in humans is being evaluated in an ongoing trial in pregnant women treated with zidovidine and randomized to receive either HIVIG or placebo (44).

A potential obstacle in developing effective prophylactic or therapeutic antibodies against HIV-1 is the paucity of available antibody reagents that effectively neutralize primary HIV type 1 (HIV-1) isolates (9, 14, 20, 29). A recent workshop on passive immunotherapy in the prevention and treatment of HIV infection, sponsored by the Pediatric AIDS Foundation, recommended that criteria for advancement of anti-HIV MAbs to clinical trials include in vitro neutralization (by >90%) of most clade B primary isolates and evidence of neutralizing activity at doses of <5 to 10 μg/ml (60). Furthermore, it was recommended that combinations of antibodies be considered, as they may act synergistically and have an improved breadth of activity against primary isolates. In this study, we evaluated the individual and combined neutralizing activities of three antibody reagents that display substantial neutralizing activity against HIV-1 primary isolates: human MAbs 2F5 and 2G12

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(5, 16) and a polyclonal hyperimmune HIVIG (17). Published studies have shown that individually, MAbs 2F5 and 2G12 neutralize most clade B primary HIV-1 isolates (16, 66, 67). However, some viruses were sensitive to neutralization by one MAb but relatively resistant to neutralization by the other (66). Similarly, preliminary data from our laboratory suggested that HIVIG neutralized primary HIV-1 isolates, although viruses varied substantially in sensitivity. To evaluate the magnitude and breadth of neutralization of these three antibody reagents alone and in combination, and to assess possible synergistic interactions, we performed neutralization experiments with a panel of 14 primary, and 1 T-cell line-adapted, clade B HIV-1 isolates.

MATERIALS AND METHODS

Antibody reagents. HIVIG (manufactured as HIV-IG by NABI, Boca Raton, Fla.) is a preparation of purified polyclonal anti-HIV immunoglobulin derived from plasma of multiple HIV-1-positive donors (17, 44). Donors are selected from geographically diverse regions of the United States, are clinically asymptomatic, have $CD4^+$ T-lymphocyte counts of $\geq$400/μl and high anti-p24 antibody titers, and are negative for p24 Ag. The product is a 50-mg/ml solution that contains 98% monomeric immunoglobulin G. Human MAbs 2F5 and 2G12 were kindly provided by Hermann Katinger (University of Agriculture, Vienna, Austria). MAb 2F5 recognizes the gp41 sequence ELDKWA that is conserved among most clade B viruses (16, 52). MAb 2G12 recognizes a conformationally sensitive epitope in the C3-V4 region of gp120 (67). Control human intravenous immunoglobulin (IVIG) was purchased from the manufacturer (Gamimune N; 5%; Miles Pharmaceutical, Elkhart, Ind.), and hepatitis B immunoglobulin (HBIG; 5%) was provided by NABI.

Virus isolates. Fourteen primary clade B HIV-1 isolates and one T-cell line-adapted virus (HIV-IIIB) were evaluated in this study. The primary isolates were arbitrarily selected from several available sources. US660, US727, US714, TH014, HT593, and HIV-IIIB were obtained from the NIH AIDS Reference and Reagent Program. US1, US2, BK132, CM237, US140, US141, US142, US143, and US144 were contributed by investigators from the U.S. Military HIV Research Program. Virus strains US140 to US144 were isolated from HIV-1-infected infants. Country designations are as follows: US, United States; TH, Thailand; BK, Bangkok, Thailand; and HT, Haiti. All primary viruses were early-passage isolates prepared by infection of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) as previously described (48, 49). Viruses BK132, HT593, and US143 had a syncytium-inducing phenotype by MT2 assay (37); all other primary viruses were non-syncytium inducing. The HIV-IIIB virus stock was prepared by infecting PBMC with supernatant from chronically infected H9 cells. All viruses were previously designated as genetic subtype B by envelope gene sequence analysis or by heteroduplex mobility assay (19, 27, 46).

gp140 binding competition studies. Real-time binding interactions between ligand (soluble oligomeric gp140 covalently linked to a biosensor matrix) and antibody reagents HIVIG, 2F5, and 2G12 was measured by surface plasmon resonance (SPR) as previously described (68–70). To measure the inhibitory effect of HIVIG on gp140 binding by 2F5 and 2G12, binding by a single concentration of 2F5 or 2G12 (10 μg/ml) was measured after serial twofold concentrations of HIVIG had been reacted with gp140 and compared to baseline binding when no HIVIG was present. For each MAb, data were displayed as percent inhibition on the *y* axis versus concentration of HIVIG on the *x* axis. These experiments were performed with gp140 from two strains of HIV-1 (IIIB and CDC-451) (38, 71).

Neutralization assay. Neutralization of virus infection of PHA-stimulated PBMC was performed as previously described, with some modifications to accommodate input of multiple antibody reagents (48, 49). For experiments with a single dose of each antibody reagent (i.e., 2F5 or 2G12, each at 25 μg/ml; HIV-IG, 2,500 μg/ml), 20 μl each of one, two, or three antibody reagents was added sequentially to triplicate wells of a 96-well culture plate. When single antibodies or double-antibody combinations were studied, phosphate-buffered saline was used to keep volumes consistent (i.e., final antibody volume, 60 μl). One hundred 50% tissue culture infective doses ($TCID_{50}$) of virus stock (20 μl) was added to antibodies and incubated for 30 min at 37°C, followed by addition of 1.5×10^5 PHA-stimulated PBMC per well (20 μl) and overnight incubation. To remove the high level of anti-p24 antibody found in HIVIG (47), cells were washed four times in 0.5-ml 96-well plates (PGC, Frederick, Md.) before transfer to a 0.2-ml round-bottom microtiter plate. PBMC were maintained in culture media containing 20 U of human recombinant interleukin-2 per ml. The final concentration of each antibody was calculated in the presence of virus, antibody, and cells (i.e., final volume of 100 μl). Neutralization of PBMC infection was assessed by measuring p24 Ag (Coulter, Miami, Fla.) expressed in culture supernatants during the early phase of virus expression (days 4 to 6, depending on the virus). In each experiment, two sets of triplicate control wells contained virus preincubated with phosphate-buffered saline (no antibody). In some experiments, additional controls included virus preincubated with irrelevant antibodies IVIG and HBIG. Dose-response data for each antibody were generated by serial twofold dilutions starting at 50 μg/ml for MAbs and 5,000 μg/ml for HIVIG. Similarly, the triple-drug combination tested serial twofold dilutions of a fixed ratio of the three antibodies, using the same starting concentrations.

Antibody interaction and synergy analysis. The antibody interaction analysis evaluated the neutralization data from the eight combinations of the three antibody reagents against each virus (i.e., single concentration of each antibody). The eight combinations included one with no antibody, three with one antibody only, three with two antibodies, and one with all three antibodies. A main effect for each individual antibody was calculated as well as two and three-way antibody interactions. The main effect is the difference in mean $\log_{10}$ p24 Ag level when an antibody is present compared to when it is absent. For example, the main effect of HIVIG on a virus is the mean of $\log_{10}$ p24 levels when HIVIG is present (i.e., HIVIG, HIVIG/2F5, HIVIG/2G12, and HIVIG/2F5/2G12) minus the mean when HIVIG is not present (i.e., no antibody, 2F5, 2G12, and 2F5/2G12). The three possible two-way interactions are the change in each main effect when an additional antibody is present, such as the change in the main effect of HIVIG upon addition of 2G12. If the main effect of HIVIG is significantly increased by 2G12, there is a positive interaction (i.e., the antibody combination produces an effect that is significantly greater than the sum of the main effects). The single three-way interaction is the change in any two-way interaction upon addition of a third antibody. All main effects and all interactions were estimated by a weighted sum of the eight mean $\log_{10}$ p24 levels, four means having weights of +1 (when antibody was present), and four having weight of −1 (25). Each two-way interaction is the difference of the two main effects, and the single three-way interaction is the difference of any two two-way interactions. A two-sided test of a null hypothesis of zero main effect, or zero interaction, was obtained by dividing an estimate by its standard deviation and referring this to a Gaussian distribution with mean 0 and standard deviation 1. Calculations of variance were based on the two to four replicate experiments for each virus. For the data from one virus, all main effects and interactions have the same variance, namely, the sum of the variances of the mean of each of the eight replicated antibody combinations. This calculation was implemented in a computer program written for this purpose. The interaction measures produced by this analysis do not directly relate to the concept of synergy described below.

Estimation of synergy was performed in two steps: (i) approximating the dose-effect response curve for the single and combination antibody reagents, using a logistic dose-response function, and (ii) calculating the synergy index (SI) that compares the predicted additive effect of the three antibody reagents to the observed effect (derived from the logistic function of the triple-combination curve). This analysis employed the COMBO program method as previously described (2, 7, 74). Neutralization effect was calculated as fraction of remaining activity (ratio of p24 Ag level with antibody present/p24 Ag level with no antibody). The dose-response curves for the individual antibodies were well described by using the logistic dose-response function $f = 1/(1 + d/k)$, where f is the effect (fraction remaining activity), d is the antibody dose, and k is an empiric binding constant. However, the data for the antibody combination were more closely fit with the two-parameter logistic function $f = 1/[1 + (d/k)^b]$. For a specified antibody dose, the analysis of synergy compares the observed inhibition of HIVIG/2F5/2G12 with a predicted effect had the three antibodies been mutually exclusive in their action. Thus, $SI = 1 - (f_o/f_p)$, where f_o is the observed effect for the triple combination (as fitted by the logistic function) and f_p is the predicted effect at the same dose based on the additive model. In this model, $f_p = 1/(1 + d1/k1 + d2/k2 + d3/k3)$, where the values $k1$, $k2$, and $k3$ were derived from the fitted individual dose-response functions for HIVIG, 2F5, and 2G12, respectively. When the observed effect is much smaller than the predicted effect, the maximum for SI is 1. The antibody dose reduction index (DRI) is the ratio of the dose predicted by the additive model to the dose producing the same level of effect based on the combination model. DRI is calculated from the SI as follows: $DRI = (1 - f_o)/(1 - f_o - SI)$. If there is no synergy, the DRI will equal 1.0. With strong synergy, the DRI may be quite large (74). The 95% confidence intervals were estimated for SI and DRI by a standard bootstrap procedure (21). For each virus, the dose-response data for each antibody individually, and for the triple combination, were sampled with replacement. The resampled data were analyzed for $k1$, $k2$, $k3$, k, and b, just as for the original experiment, leading to new estimates of SI and DRI. This process was repeated 300 times. The upper and lower 2.5 percentiles of the SI and DRI values estimate the respective 95% confidence intervals.

RESULTS

Binding competition studies. MAbs 2G12 and 2F5 have distinct binding sites on gp120 and gp41, respectively, and as expected, SPR binding studies revealed no significant competition between the two MAbs for binding to gp140 at concentrations as high as 25 μg/ml (data not shown). Since HIVIG is a polyclonal product that likely has a diverse array of antibodies to HIV-1 envelope glycoprotein, we next studied if 2F5 and 2G12 could bind to gp140 that had first been reacted with

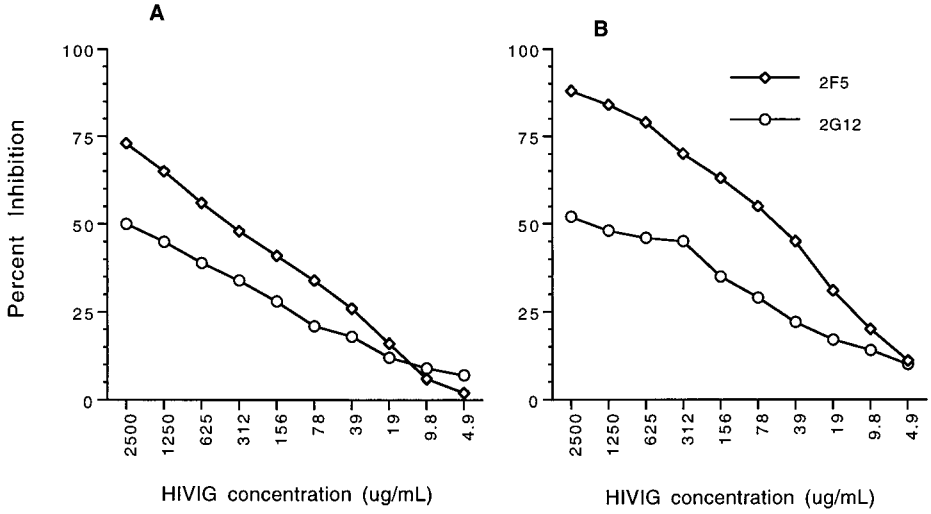

FIG. 1. Percent inhibition of binding of MAbs 2F5 and 2G12 to gp140 from strain CDC-451 (A) and strain IIIB (B) measured by SPR. Total binding of 2F5 and 2G12 (each at 10 μg/ml) was measured separately against each gp140. Results were compared to MAb binding after serial twofold dilutions of HIVIG had been bound to gp140 and are expressed as percent inhibition of MAb binding at each dilution of HIVIG.

increasing doses of HIVIG. As shown in Fig. 1, HIVIG partially inhibited binding of each MAb to gp140. However, even at the highest HIVIG concentration of 2,500 μg/ml, the inhibition was incomplete. Maximum inhibition of 2G12 binding was 50% or less, while 2F5 inhibition was substantially higher (73 and 88% on gp140 from strains CDC-451 and IIIB, respectively).

Neutralization experiments. To evaluate the individual and combined neutralization effects of HIVIG, 2F5, and 2G12 against a panel of primary isolates, initial experiments used a single dose of HIVIG (2,500 μg/ml) and each MAb (25 μg/ml). These antibody concentrations were chosen because they are in the middle to upper range of what can be achieved in vivo after passive administration (11, 15, 17, 31, 33, 75) and display significant in vitro neutralization (i.e., ≥90%) against most viruses (66). Table 1 summarizes the neutralization data for the antibody combinations against 15 HIV-1 isolates, expressed as $\log_{10}$ reductions in p24 Ag. Due to technical limitations, not all antibody combinations were evaluated in each experiment. As an example of individual experiments, Fig. 2 shows the p24 Ag data from a single set of experiments with eight viruses. HIVIG, 2F5, and 2G12 were tested alone and compared to the double combination 2F5/2G12 and triple combination HIVIG/2F5/2G12. Individual reagents often produced a 10-fold or greater reduction in p24 Ag (i.e., ≥90% neutralization) compared to control containing no antibody. Against all eight viruses, the greatest p24 Ag reduction occurred in the presence of all three antibody reagents (range, 2 to 5 $\log_{10}$). For three viruses (93US140, BK132, and 92HT593), no p24 Ag was detected in culture supernatants of PBMC after virus was preincubated with HIVIG/2F5/2G12 (limit of sensitivity of Coulter enzyme-linked immunosorbent assay, 7.8 pg/ml). The value of 1 pg/ml shown in Fig. 2 was assigned only after the culture was monitored for 21 days and no p24 Ag was detected.

To estimate if the combination HIVIG/2F5/2G12 could consistently prevent infection of mitogen-stimulated PBMC by ~100 $TCID_{50}$ of virus, three independent experiments were performed with BK132 and HT593. Viral p24 Ag was measured in culture supernatants every 3 to 4 days for 21 days. No

TABLE 1. Neutralization values for antibody reagents against 15 clade B HIV-1 isolates

Antibody reagent[b]	$\log_{10}$ decrease in p24 antigen[a]															Mean[c]	Total[d]
	US1	US2	US660	BK132	HT593	CM237	TH014	US727	US714	US140	US141	US142	US143	US144	IIIB		
IVIG	0.1	−0.1	0.0	0.2	0.1	0.1	−0.1	−0.1	0.0	0.0	−0.1	0.0	0.0	−0.2	0.0	0.0	0/15
HBIG	0.1	0.1	−0.2	0.2	0.1	0.2	0.0	0.0	0.1	−0.1	−0.2	−0.1	0.1	−0.2	0.1	0.0	0/15
HIVIG	0.7	0.6	0.3	0.9	0.9	1.1	0.6	0.9	0.4	0.4	0.5	0.8	0.6	0.5	1.7	0.7	2/15
2F5	1.1	1.2	1.0	0.7	0.8	1.4	0.7	0.2	0.5	0.6	1.2	1.5	0.6	1.1	1.7	1.0	8/15
2G12	1.4	0.3	1.2	1.6	1.1	0.8	1.0	2.8	0.0	1.3	0.6	0.4	0.8	1.2	1.3	1.1	9/15
HIVIG/2F5	1.8	1.6	1.5	1.5	1.9	2.2	0.9	1.0	ND	ND	ND	ND	ND	ND	ND	1.6	7/8
HIVIG/2G12	1.7	0.8	2.1	2.4	1.9	2.0	1.1	3.3	ND	ND	ND	ND	ND	ND	ND	1.9	7/8
2F5/2G12	1.9	1.4	2.3	2.7	2.0	1.9	1.7	nd	0.9	1.9	1.9	2.0	1.2	1.7	2.5	1.9	13/14
HIVIG/2F5/2G12	2.8	2.2	2.7	5.2	4.3	2.5	2.1	4.3	0.9	4.4	2.8	2.4	1.8	2.4	4.2	3.0	14/15

[a] Mean of two to four independent experiments. ND, not determined.
[b] Antibody reagents are described in Materials and Methods. Concentrations: IVIG, HBIG, and HIVIG, 2,500 μg/ml; MAbs 2F5 and 2G12, 25 μg/ml.
[c] Mean neutralization value of each antibody reagent against all 15 virus isolates.
[d] Total number of viruses neutralized (≥1.0 $\log_{10}$ p24 decrease)/total tested.

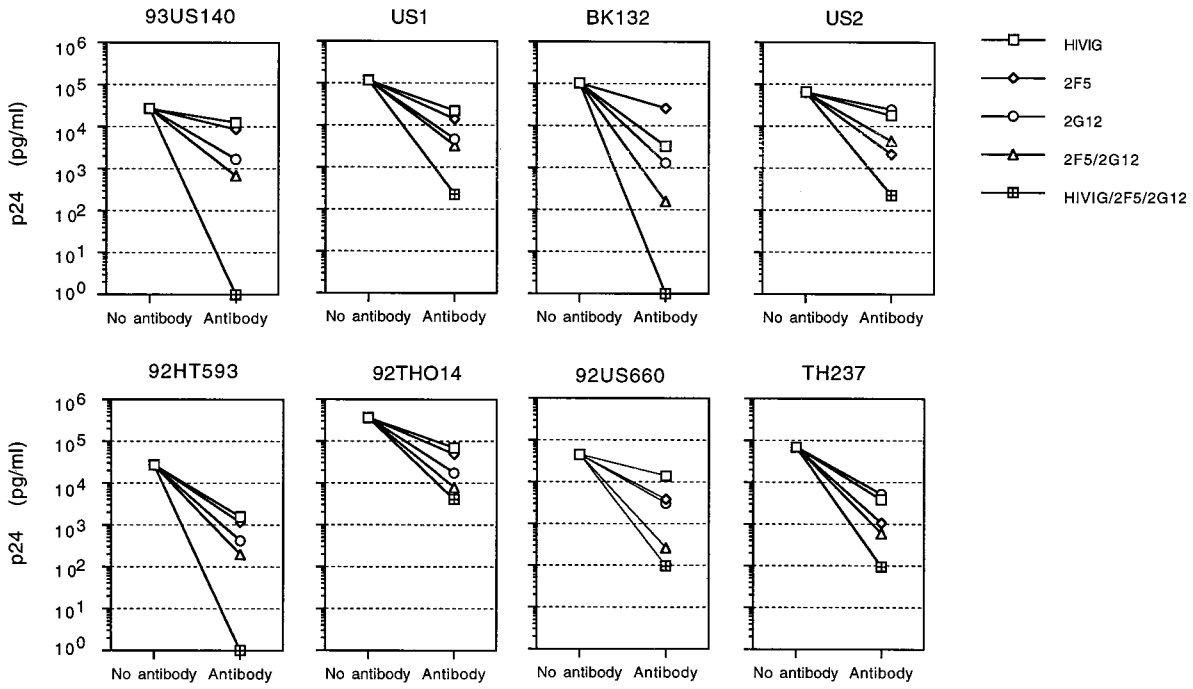

FIG. 2. Individual neutralization experiments with antibody reagents HIVIG, 2F5, 2G12, the double combination 2F5/2G12, and the triple combination HIVIG/2F5/2G12. The HIVIG concentration was 2,500 μg/ml, and MAb concentrations were 25 μg/ml. The no-antibody lane indicates p24 Ag level when no antibody was present. A value of 1 pg/ml was assigned if no p24 Ag was detected through 21 days of culture.

p24 Ag was detected in the three experiments with BK132; low levels of p24 Ag (<1 ng/ml) were detected in one of the three assays with HT593. Figure 3 displays virus growth kinetics for one experiment with BK132 and HT593. Individual antibody reagents and the combinations HIVIG/2F5 and HIVIG/2G12 clearly reduced p24 expression during the initial round of virus replication (first several days), but some virus was able to infect PBMC and propagate in culture (indicated by delayed replication kinetics compared to the no-antibody control). However, no p24 Ag (BK132), or very low levels (i.e., HT593 in one of three experiments), was detected from PBMC infected by virus preincubated with the triple-antibody combination. Similar complete inhibition of PBMC infection was seen against US727, US140, and IIIB (in two of two experiments [data not shown]). Of the 15 viruses tested, the triple-antibody combination could completely neutralize infection by five isolates: BK132, HT593, US727, US140, and IIIB. This effect was not seen with any double-antibody combination.

As summarized in Table 1, a single dose of each individual antibody, the double-MAb combination 2F5/2G12, and the triple-antibody combination were assayed against all 15 viruses. In addition, all eight possible antibody combinations (no antibody, single antibody, 2F5/2G12, HIVIG/2F5, HIVIG/2G12, and HIVIG/2F5/2G12) were evaluated against the first

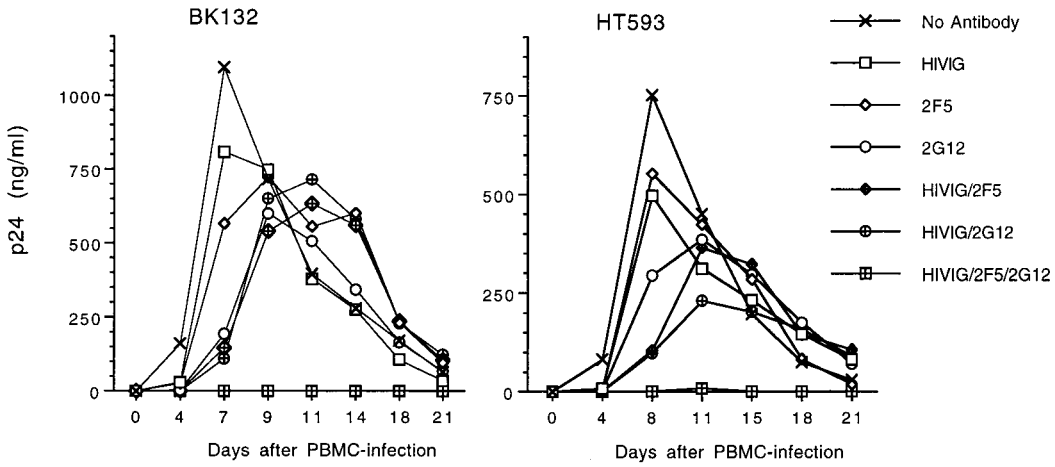

FIG. 3. Virus growth kinetics for isolates BK132 and HT593 after neutralization with antibody reagents, individually or in combination. Half of the culture medium was replaced every 3 to 4 days.

TABLE 2. Estimates of antibody main effect and two-way and three-way interactions[a]

Antibody reagent	Log_{10} decrease in p24 Ag						
	US1	US2	US660	BK132	HT593	CM237	TH014
HIVIG	−0.72	−0.80	−0.61	−1.43	−1.30	−0.89	−0.58
2F5	−0.89	−0.72	−1.26	−1.78	−1.29	−0.93	−0.74
2G12	−1.04	−0.57	−1.02	−1.53	−1.35	−0.77	−0.63
HIVIG/2F5	−0.09	0.18	−0.34	−1.13	−0.49	0.36	−0.20
HIVIG/2G12	−0.02	−0.55	0.18	0.09	−0.28	−0.08	0.12
2F5/2G12	0.21	0.19	0.19	−0.46	−0.30	0.20	0.08
HIVIG/2F5/2G12	−0.13	−0.12	0.15	−0.48	−0.34	0.10	−0.14

[a] Main effect and two- and three-way interaction analysis and meaning of negative values are described in Materials and Methods. Underlined values are significantly different from zero ($P < 0.05$).

seven viruses shown. The results are displayed as log_{10} decrease in p24 Ag, and values of ≥1.0 (i.e., ≥90% neutralization) were considered evidence of effective virus neutralization. Irrelevant polyclonal antibody IVIG and HBIG did not significantly reduce p24 Ag values. Individual antibody reagents displayed some neutralization against many of the 15 viruses tested, but there was significant variation in neutralization potency among the viruses. HIVIG, 2F5, and 2G12 resulted in ≥90% neutralization against 2, 8, and 9 of 15 viruses, respectively. Compared to individual antibody reagents, the combination of HIVIG plus MAb (2F5 or 2G12) neutralized 7 of 8 viruses, and the combination of two MAbs (2F5/2G12) neutralized 13 of 14 viruses. As shown is the last two columns of Table 1, the mean magnitude of neutralization of the double combinations was greater than for individual antibody reagents, and the triple combination produced the greatest magnitude and breadth of neutralization. HIVIG/2F5/2G12 neutralized 14 of 15 viruses, with a mean effect of 3.0 log_{10} (i.e., 99.9% neutralization)

Analysis of antibody interaction and synergy. An antibody interaction analysis was possible when all eight antibody combinations were represented. We were therefore able to evaluate antibody interactions for US1, US2, US660, BK132, HT593, CM237, and TH014. For each virus, the main effect of a single antibody reagent was the difference in effect (measured as mean log_{10} p24 Ag level) when an antibody was present compared to the effect when it was absent, averaged for all combinations. Thus, a negative value indicated a log_{10} reduction in p24 Ag in the presence of the antibody. Two- and three-way interactions were also calculated as described in Materials and Methods. Table 2 shows the main effects and two- and three-way interactions for the seven viruses noted above. As an indication of neutralizing activity, the main effect of each individual antibody against all seven viruses was greater than 0.5 log_{10}, and all values were statistically significant. As evidence of positive two-way antibody interactions, all of the double combinations (i.e., HIVIG/2F5, HIVIG/2G12, and 2F5/2G12) displayed a significant positive interaction against at least two of the seven viruses (underlined negative values). Significant three-way interaction were seen for viruses BK132 and HT593 (log10 p24 values of −0.48 and −0.34, respectively; P values, 0.016 and 0.011). Of note, double- and triple-antibody combinations that do not have statistically significant interaction values (those not underlined) produce neither a positive nor a negative interaction, and therefore the effect is simply the sum of the two or three main effects. One antibody combination (HIVIG/2F5) demonstrated a negative interaction against one virus (CM237), indicating an effect that is less than the sum of the two main effects of these two reagents for this virus.

While combinations of single doses of antibody reagents allow an interaction analysis, calculations of antibody synergy require antibody dose-response curves. We therefore evaluated the neutralization effects of serial two-fold dilutions of HIVIG, 2F5, and 2G12, individually and as a fixed triple combination, against four viruses. Starting concentrations were 5,000 μg/ml for HIVIG and 50 μg/ml for 2F5 and 2G12. Figure 4 shows the dose-response curves and again demonstrates the potent neutralization by the HIVIG/2F5/2G12 combination. The data shown are from one of two independent experiments with similar results. Double-antibody combinations were not assayed in this format. Each of the dose-response curves shown in Fig. 4 was fit with a two-parameter logistic function as described above. At a specified antibody dose, the analysis of synergy compared the inhibition of HIVIG/2F5/2G12 described by the logistic function with a predicted additive effect had the three antibodies been mutually exclusive in their action. Since the SI is 1 minus the ratio of the observed to predicted effects, when the observed effect is the same as the predicted (additive) effect, the SI equals zero and there is no synergy. Similarly, when the observed effect is much smaller than the predicted effect, the maximum value for SI is 1. As an example, fitting the data for the virus US1 in Fig. 4 gives the following k values (micrograms per milliliter): HIVIG, 272; 2F5, 1.24; 2G12, 1.45; and triple combination, 1.41. Based on the two-parameter logistic fit of the combination data, the fraction inhibition (f_o) = 0.0025 at an antibody dose of 50 μg/ml (5,000 μg/ml for HIVIG), whereas the predicted effect (f_p) = 1/(1 + 5,000/272 + 50/1.24 + 50/1.45) = 0.011. Thus, SI = 1 − (0.0025/0.011) = 0.76 (as shown for US1 in Table 3). The associated DRI of 4.2 indicates that approximately four-

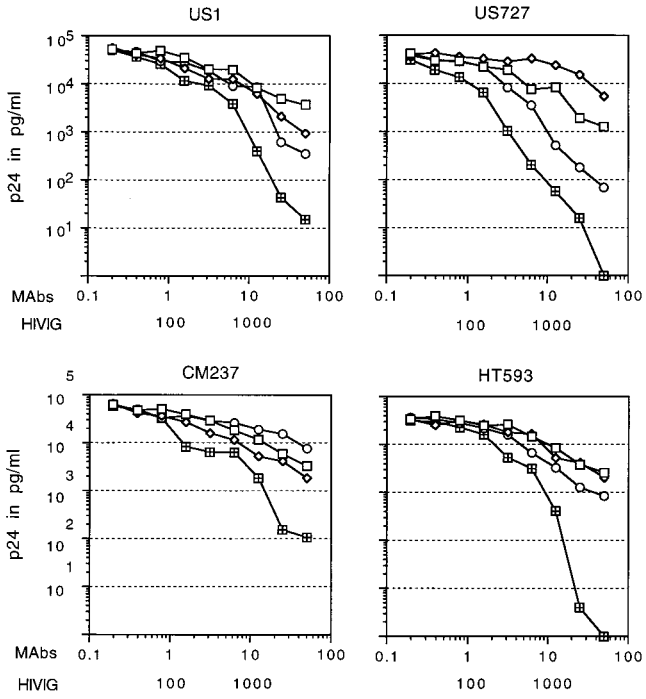

FIG. 4. Neutralization dose-response curves for HIVIG (□), 2F5 (◇) 2G12 (○), and the triple combination HIVIG/2F5/2G12 (⊞). Values for MAbs and HIVIG on the x axis are in micrograms per milliliter.

TABLE 3. SI and DRI at multiple antibody doses[a]

MAb dose[b] (μg/ml)	US1		US727		HT593		CM237	
	SI	DRI	SI	DRI	SI	DRI	SI	DRI
50	0.76	4.2	0.93	13.7	0.76	4.2	0.53	43
25	0.68	3.2	0.90	10.1	0.70	3.1	0.52	42
12.5	0.57	2.4	0.87	7.5	0.57	2.4	0.51	15
6.25	0.43	1.9	0.81	5.4	0.42	1.8	0.50	11
3.12	0.24	1.4	0.73	4.0	0.23	1.4	0.47	2.8
1.56	0.00	1.0	0.59	3.5	0.03	1.1	0.42	1.6

[a] SI and DRI are calculated as described in Materials and Methods. Underlined values are statistically significant by bootstrap analysis.

[b] MAb concentration in the triple combination HIVIG/2F5/2G12. The corresponding HIVIG concentration was 100-fold higher than the MAb concentration.

fold less of each antibody is required in the antibody mixture compared to the concentration of antibody alone to give the same neutralization effect. Table 3 displays the SI and DRI at multiple doses of the triple-antibody combination and shows that the greatest level of synergy, and concomitant larger DRI values, were demonstrated at the higher doses of antibody. Underlined values indicate that the null hypothesis of SI = 0 is excluded by the bootstrap analysis.

DISCUSSION

We studied the combination of three antibody reagents that each displayed moderately potent neutralization of primary HIV-1 isolates. While two MAbs with distinct binding sites on gp160 might be expected to produce additive or possibly synergistic neutralization, the interaction of MAbs with a polyclonal product was less intuitive. SPR studies of MAb binding to recombinant gp140 confirmed that 2G12 and 2F5 did not cross-compete for binding to sites on gp120 and gp41, respectively. Of greater interest was the interaction of HIVIG and the MAbs (Fig. 1). The ability of 2G12, and to a lesser extent 2F5, to bind to gp140 after relatively high doses of HIVIG were bound suggested that antibodies to the 2G12 and 2F5 binding sites were not prominent in HIVIG. Therefore, the MAbs might produce additional virus neutralization when combined with HIVIG. This was demonstrated in neutralization studies with a single dose of each antibody that evaluated the interaction among all eight antibody combinations. As shown in Tables 1 and 2, negative antibody interactions were rarely seen. Only the two-way interaction of HIVIG and 2F5 against virus CM237 displayed a statistically significant effect that was less than the sum of each antibody's main effect (Table 2; value of 0.36). Estimates of all other two- and three-way antibody interactions produced neutralization equal to or greater than the sum of individual antibody main effects. The triple combination of antibodies produced the greatest magnitude and breadth of neutralization and neutralized by ≥95% 14 of 15 viruses tested.

Synergistic neutralization by the triple-antibody combination was demonstrated by analysis of the dose-response neutralization data displayed in Fig. 4. We estimated synergy based on the concept of an SI that is similar to the combination index initially devised to describe multiple drug effects at a single site and often applied to MAb neutralization studies (8, 13, 40, 43, 50, 51, 64, 72). Our studies included two MAbs that bind to different sites on HIV-1 gp160 and a polyclonal reagent with a diverse spectrum of anti-gp160 antibodies. This produces a more complex set of interactions than can be readily modeled without a more extensive data set. However, an advantage of our approach is that the dose-response curves were fit with a two-parameter logistic function that produces a better fit of the data than a linear log-log dose-response plot. As seen in Fig. 4, the neutralization effect of the individual antibodies, and the triple combination, was most pronounced at the higher concentrations tested. This is also shown in Table 3, where the SI and DRI increase with increasing antibody dose. While we should be cautious in extrapolating in vitro data to in vivo effect, this finding suggests that combinations of neutralizing antibodies would have their most pronounced synergistic effect at higher doses. The dose-response curves and synergy analysis was only performed for the triple-antibody combination. As discussed above, the single-dose combinations of two antibodies often produced a neutralization effect that was greater than that predicted by the effect of each of the individual antibodies. Additional studies of the dose-response curves of double-antibody combinations would be required to determine if double combinations of HIVIG, 2F5, and 2G12 would produce synergistic neutralization.

In several of the experiments shown, the triple-antibody combination appeared to completely prevent infection of PBMC by 100 $TCID_{50}$ of virus (Fig. 2 to 4). This effect was reproducible and, for two viruses studied (BK132 and HT593), occurred even at a virus input of 1,000 $TCID_{50}$ (data not shown). How this impressive in vitro neutralization (i.e., neutralization of >99.9% of infectious virus) would translate to in vivo neutralization is not clear. The in vitro assay preincubates antibody and virus, uses mitogen-stimulated $CD4^+$ target cells, and washes out antibody after target cell infection. This is designed to measure the fraction of infectious virus neutralized and not the effect of antibody on subsequent rounds of virus replication. Thus, there are limitations to extrapolating an in-vitro assay designed to demonstrate an effect with in vivo virus neutralization. Nonetheless, if the in vitro measurement of neutralization has some physiologic relevance, the comparison of antibody combinations and the measure of breadth of neutralization may be important predictors of antibody effect.

Our data indicate that when used alone, two potent anti-HIV-1 MAbs, or HIVIG derived from multiple healthy donors, can potently neutralize many, but not all, clade B isolates. This contrasts with a product such as HBIG that is highly effective in neutralizing the vast majority of hepatitis B viruses worldwide (due to a highly conserved neutralization epitope on hepatitis B virus) (62). Therefore, there may be important advantages to passive immunotherapy with an HIV-1 antibody cocktail that displays a greater magnitude and breadth of neutralization than the individual reagents. The antibody combination may extend benefit to a greater number of patients and may reduce the dose requirement and therefore the cost of passive antibody administration. While only clade B viruses were tested in this study, an evaluation of these antibody combinations against non-clade B viruses is ongoing. Trkola et al. have shown that MAbs 2F5 and 2G12 can neutralize across HIV-1 subtypes, and we might therefore expect to see similar additive or synergistic effects against a more diverse panel of viruses (66). However, there are significant limitations in the breadth of activity of both MAbs. In the report by Trkola et al., MAb 2G12 neutralized three of three clade A, one of three clade C, three of four clade D, none of three clade E, one of three clade F, and none of two group O viruses (defined as a 90% inhibitory dose of ≤50 μg/ml). MAb 2F5 was more broadly reactive but becomes inactive with amino acid changes within the central core of its ELDKWA epitope (10, 66). Similarly, the breadth of effect of a clade B HIVIG against non-clade B viruses is not yet well described. Thus, passive administration of an antibody combination that is broadly active

against HIV-1 strains worldwide may require further development of potent neutralizing antibody reagents including MAbs and/or HIVIG derived from non-clade B HIV-1-infected subjects.

There have been prior reports of synergistic HIV-1 neutralization (1, 6, 8, 18, 40, 43, 50, 51, 55, 63, 64), including a recent study by Vijh-Warrier et al. that demonstrated neutralization by a three-MAb combination (72). Our data extend these observations by evaluating a large panel of clade B viruses and by using three antibody reagents with demonstrated neutralizing activity against primary HIV-1 isolates. Thus, this is the first report of synergistic neutralization of a panel of primary HIV-1 isolates and the first report to evaluate the interaction of MAbs 2F5 and 2G12 with HIVIG. The mechanism of synergistic interactions among these three antibody reagents, one of which is polyclonal, is likely to be complex. MAb 2G12 inhibits the interaction of gp120 with the β-chemokine receptor CCR5 (65), while 2F5 appears to affect the conformation of the gp41 fusion domain and thus inhibit virus-cell fusion (53, 58). The synergistic effect seen in our studies is likely a consequence of the complementary activity of these two MAbs, together with the functionally diverse spectrum of antienvelope antibodies present in HIVIG. The improved magnitude and breadth of neutralization demonstrated by combining neutralizing MAbs with HIVIG suggests that such antibody combinations may be more effective than individual agents when used as passive immunotherapy for HIV-1. As passive administration of HIVIG has been studied in the treatment of ongoing HIV infection, and is currently being studied as a method of interrupting maternal-infant transmission, future studies should consider combinations of HIVIG and potent neutralizing MAbs.

ACKNOWLEDGMENTS

We thank Sonja Surman, Susan Hegerich, Steven Veit, and Robert Kaminski for excellent technical assistance; Jean Carr, Larry Loomis-Price, and Bruce Gilliam for critical review of the manuscript; and Donald S. Burke for helpful discussions and support of this work.

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

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