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-IIIIB, 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 zidovudine 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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and cells (i.e., final volume of 100 μl) and the concentration of each antibody was calculated in the presence of virus, antibody, and high anti-p24 antibody found in HIVIG (47). Cells were preincubated with phosphate-buffered saline (no antibody). In some experiments, additional controls included virus precipitated with irrelevant antibodies HIVIG and HBIG. Dose-response data for each antibody were generated by serial twofold dilutions starting at 50 μg/ml for HBIG 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 antibody 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, 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. 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/ks) where k is the fraction (removal 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/ks)^1/k] for a specified antibody dose. The application of the synergy analysis compares the observed inhibition of HIVIG/2F5/2G12 with a predicted effect that has been equal to the observed effect for the triple combination (as fitted by the logistic function) and f is the predicted effect at the same dose based on the additive model. In this model. f = 1 - 3/(d/k1 + d/k2 + d/k3), where the values k1, k2, and k3 were derived from fitted individual dose-response curves and k is directly related to the maximum for SI = 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)/f where f = 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).

RESULTS

Binding competition studies. MAbs 2G12 and 2F5 have distinct binding sites on gp120 and gp41, respectively, and as expected, SPR binding revealed negative binding 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 antibody 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...
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 log10 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 log10). 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 TCID50 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

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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 log10 p24 decrease)/total tested.
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
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 (2F5 or 2G12) neutralized 7 of 8 viruses, and the combination of two MAb (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 therefore were 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 of 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 (log_{10} 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 the three antibodies had 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 (I) = 0.0025 at an antibody dose of 50 μg/ml (5,000 μg/ml for HIVIG), whereas the predicted effect (I) = 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-
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 antibody 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 clade B viruses is not yet well described. Thus, passive administration of HIVIG against non-clade viruses (66). However, there are significant limitations in the breadth of activity of both MAbs. In the report by Trkola et al., we showed that MAbs 2F5 and 2G12 can neutralize across 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 compared to the concentration of antibody alone to give the same neutralization effect. Table 3 displays the SI and DRI at multiple antibody 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.

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<th>US1 SI</th>
<th>US1 DRI</th>
<th>US727 SI</th>
<th>US727 DRI</th>
<th>HT593 SI</th>
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*SI and DRI are calculated as described in Materials and Methods. Underlined values indicate that the null hypothesis of SI = 0 is excluded by the bootstrap analysis.

* MAb concentration in the triple combination HIVIG/2F5/2G12. The corresponding HIVIG concentration was 100-fold higher than the MAb concentration.
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 Vijd-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. MAP 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 anti-Env 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 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 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 immunotherapy for HIV-1.


