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Synergistic Inhibition of Human Immunodeficiency Virus Type 1 Envelope Glycoprotein-Mediated Cell Fusion and Infection by an Antibody to CD4 Domain 2 in Combination with Anti-gp120 Antibodies

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Antibodies to several epitopes of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (gp120-gp41) can synergize in inhibiting HIV-1 infection. In the present study we tested the ability of a monoclonal antibody (MAb), 5A8, which interacts with CD4 domain 2, and other CD4-specific MAbs to synergize with antibodies against gp120. We have previously found that 5A8 inhibits HIV-1 entry without interfering with gp120 binding to CD4, presumably by affecting a postbinding membrane fusion event. Because antibodies to the gp120 V3 loop also affect post-CD4-gp120-binding events, 5A8 was first tested in combination with anti-V3 loop antibodies for possible synergy. The anti-V3 loop antibodies 0.5β, NEA-9205, and 110.5 acted synergistically with 5A8 in inhibiting syncytium formation between gp120-gp41- and CD4-expressing cells. A human MAb to an epitope of gp120 involved in CD4 binding, IAM 120-1B1, and another anti-CD4 binding site antibody, PC39.13, also exerted synergistic effects in combination with 5A8. Similarly, an antibody against the gp120 binding site on CD4, 6H10, acted synergistically with an anti-V3 loop antibody, NEA-9205. However, a control anti-CD4 antibody, OKT4, which does not significantly inhibit syncytium formation alone, produced only an additive effect when combined with NEA-9205. Serum from HIV-1-infected individuals, which presumably contains antibodies to the V3 loop and the CD4 binding site, exhibited a strong synergistic effect with 5A8 in inhibiting infection by a patient HIV-1 isolate (0104B) and in blocking syncytium formation. These results indicate that therapeutics based on antibodies affecting both non-gp120 binding and gp120 binding epitopes of the target receptor molecule, CD4, could be efficient in patients who already contain anti-gp120 antibodies and could also be used to enhance passive immunization against HIV-1 in combination with anti-gp120 antibodies.

Antibodies to several epitopes of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (the gp120-gp41 complex) can synergistically block infection and fusion mediated by gp120-gp41 (2, 5, 17, 20, 21, 24, 25, 27, 28). The use of synergistically acting antibodies can be important not only for passive immunotherapy of HIV-1 infections but also in the design of better vaccines (17). Therefore, finding antibodies to new epitopes which can synergize may prove helpful in the prevention and treatment of HIV-1 infections. Such studies might also shed light on the interaction between gp120-gp41 and CD4, which leads to membrane fusion and delivery of the viral genome into the cell. While all previous studies of synergistic antibodies focused on their interaction with gp120-gp41, here we examined the synergy between antibodies to the HIV-1 envelope glycoprotein and the antibodies to its receptor, CD4.

Monoclonal antibodies (MAbs) that bind CD4 are very potent inhibitors of HIV-1 infection and fusion (7, 16, 19). However, in spite of their high potency, anti-CD4 MAbs have received relatively little attention as a potential AIDS therapy (8). A major concern has been the possibility for suppression of immune responsiveness by the CD4-specific MAbs, which may follow after the clearance of the peripheral blood $CD4^+$ cells (14) or the downmodulation of CD4 from their surfaces (8, 14). However, it has been shown that antibodies to self-CD4 can be generated in vivo without leading to immune dysfunction (4, 29, 30). It has also been recently demonstrated that the MAb 5A8, which binds to domain 2 of the CD4 molecule and potently inhibits HIV-1 infection (3), did not induce significant loss of $CD4^+$ cells or immunosuppression in rhesus monkeys (26).

In the present study we chose the 5A8 MAb on the basis of its unique properties and tested its activity against HIV-1 envelope glycoprotein-mediated syncytium formation and infection in combination with anti-gp120-gp41 antibodies. Our results indicate that cocktails of anti-gp120 MAbs and certain anti-CD4 MAbs could be used as efficient anti-HIV-1 agents.

MATERIALS AND METHODS

Cells, virus, and reagents. The human cell lines CEM and Molt-3 were obtained from the American Type Culture Collection, Rockville, Md. The $CD4^-$ subclone 12E1 was derived from CEM cells by ethyl methanesulfonate mutagenesis and by negative selection with OKT4A with complement as previously described (13) and generously provided by Hana Golding (Food and Drug Administration, Bethesda, Md.). These cell lines were propagated in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. The human $CD4^+$ T-cell line C8166, chronically HIV-1 strain IIIB-infected H9 cells, and antiserum pooled from HIV-1-infected individuals were gifts of Robert T. Schooley (Colorado Medical School, University of Colorado, Denver). The HIV-1 neutralizing serum used in the experiments with cells expressing recombinant vaccinia virus was provided by Luba Vujcic (Food and Drug Administration); this serum is now available through the NIH AIDS Research and Reference Reagent Program (catalog no. 1983).

Primary isolate 0104B derived from clinical blood samples was propagated in normal human peripheral blood mononuclear cells activated for 2 to 3 days with phytohemagglutinin. The recombinant vaccinia virus vPE16, encoding the IIIB

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gp120-gp41 (10), was provided by P. Earl and B. Moss (National Institute of Allergy and Infectious Diseases, Bethesda, Md.) and is also available through the NIH AIDS Research and Reference Reagent Program.

Murine anti-human CD4 MAbs 6H10 and 5A8 have been previously described (3). 6H10 recognizes the gp120 binding site on CD4 domain 1, and 5A8 interacts with a non-gp120 binding epitope involving CD4 domain 2. The murine MAb OKT4, which binds to human CD4 domains 3 and 4, was purchased from Ortho Diagnostics (Raritan, N.J.). The murine anti-gp120 V3 loop MAbs NEA-9205 and 110.5 were purchased from DuPont NEN Research Products (Boston, Mass.) and Genetic Systems (Redmond, Wash.), respectively, and 0.5β was obtained through the NIH AIDS Research and Reference Reagent Program (contributed by Shuzo Matsushita). The human anti-gp120 MAb IAM 120-1B1, which blocks CD4 binding, was purchased from Viral Testing Systems (Houston, Tex.). The rat anti-gp120 MAb PC39.13, which also blocks CD4 binding, was generously provided by Jane McKeating (University of Reading, Reading, United Kingdom).

Syncytium formation assay. Syncytium formation between chronically HIV-1 IIIB-infected H9 cells and uninfected $CD4^+$ C8166 cells was produced as previously described (3). Briefly, 5×10^3 H9 cells were incubated in 0.1 ml of RPMI 1640 supplemented with a solution containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 6.8), 2 mM glutamine, and 20% fetal calf serum with purified anti-gp120 antibody for 30 min at 37°C. Then, 15×10^3 $CD4^+$ C8166 cells preincubated with purified anti-CD4 MAb for 30 min at 37°C in 0.1 ml of RPMI 1640 were added to each well for a total of 0.2 ml per well. Samples were incubated in 96-well plates at 37°C in a 5% CO_2 atmosphere, and syncytia were counted after 2 h and after 24 h. Syncytia were defined as giant cells at least four times the diameter of uninfected single cells (18). They are bound by a single membrane and are not disrupted by pipetting. In some experiments, to distinguish between syncytia and cell aggregates, we withdrew and then reinserted 0.1 ml of cell suspension 10 times with an Eppendorf pipette. This action completely disrupted the aggregates, and the syncytia were easily counted. The experiments were performed in triplicate wells and the mean numbers of syncytia were calculated. Data are expressed as the fraction inhibition, calculated as $1 - a/b$, where a is the number of syncytia in a well with antibodies and b is the number of syncytia in a well without antibodies. All the experiments were repeated at least twice, and the synergistic effect was reproducible.

The experiments with 12E1 cells expressing gp120-gp41 after infection with recombinant vaccinia virus (vPE16) were performed in a way similar to that described above (see also reference 9). However, the ratio of the gp120-gp41-expressing cells to the target $CD4^+$ Molt-3 cells was 1:1 in all experiments, and the total number of cells in 0.2 ml of RPMI 1640 medium was 10^5.

Inhibition of HIV-1 infection. Infectivity assays were carried out as previously described (3). Briefly, phytohemagglutinin-blasted peripheral blood mononuclear cells were pretreated with anti-CD4 MAbs, and the virus (primary isolate 0104B) was pretreated with a serum pool from HIV-1-infected individuals for 30 min at 37°C and then mixed with target cells. After overnight incubation, the cells were washed and the medium with MAbs and serum was replaced. Every 3 to 4 days during 2 weeks of culturing, supernatants were removed for measurement of soluble HIV-1 p24 antigen and fresh medium containing MAbs, but not antiserum, was added. The results are the averages of two independent experiments, each with duplicate culture wells. The infected cultures were counted after 2 weeks, and the fraction inhibition was calculated as $1 - a/b$, where a is the number of infected cultures in the presence of antibodies and b is the number of infected cultures without antibodies.

Quantitation of synergy. We used the concept of the combination index (CI) (28) to quantitate synergistic effects. It has been shown that for a combination of agents that do not interact with each other and therefore produce an additive effect, the sum of the ratios of their concentrations in the mixture (c_{mixt}) to the concentrations of agents that individually have the same effect as the mixture (c_{effect}) is 1 (1). This sum is the CI. When this sum is lower than 1, the agents act in synergy. For a two-component system, as in the present study, CI is calculated as $c_{1mixt}/c_{1effect} + c_{2mixt}/c_{2effect}$, where c_{1mixt} is the concentration of the first component in the mixture which leads to a certain level of inhibition (f), $c_{1effect}$ is that concentration of the first component which alone (in the absence of the second component) will result in the same inhibitory effect as the mixture of the two components, and c_{2mixt} and $c_{2effect}$ are the corresponding concentrations for the second component. For the so-called mutually nonexclusive inhibitors, a third term equal to $(c_{1mixt}/c_{1effect})(c_{2mixt}/c_{2effect})$ is added to the CI (6). For our data this term was >10-fold less than the sum of the other two terms. Calculations with this term and without it gave essentially identical results.

Calculation of the CI is performed in two steps: (i) approximating the dose-effect response for each drug by empirical functions and (ii) calculating the CI on the basis of information derived from those functions. To describe quantitatively the dose-response effect, we initially used the following approximating function: $f = c^m/(k + c^m)$, where f is the effect (fraction inhibition), c is the dose (antibody concentration), and k and m are empirical constants, as suggested by the classic method of Chou and Talalay (6). However, the fitting of our data by this function was not very good; in some cases the linear correlation coefficient was below 0.9. Therefore, we used a more complex function to fit the data: $f = [c^m/(k + c^m)]^n$, where n is another empirical constant. With this function we produced results that better approximated our data (the correlation coefficients were higher than 0.9).

The fraction inhibition was calculated as $1 - a/b$. The constants k, n, and m were calculated by fitting the data with the computer program SigmaPlot. Then, the CI was calculated as $c_{1mixt}/c_{1effect} + c_{2mixt}/c_{2effect}$, where the concentrations ($c_{1,2effect}$) of the agents acting alone to produce the fraction of inhibition of the mixture were calculated by the formula with the predetermined empirical constants. We calculated all CIs by using Chou and Talalay's approach (6) with the computer program for dose-effect analysis (Biosoft, Cambridge, United Kingdom) and compared the results with our results obtained by the more complex fitting function. Interestingly, in most cases we obtained very similar values for the CI, in spite of the low correlation coefficients obtained by fitting our data with the computer program purchased from Biosoft. One advantage of the more complex formula compared with the computer program, in addition to its high accuracy, is the possibility to use data with a zero concentration and a fraction inhibition equal to 1. We found that in some cases, using these values is important for obtaining accuracy of fitting of the data. The CIs presented in this paper were calculated on the basis of the more complex function for reasons of caution. One example of calculation by this method follows.

Fitting the data shown in Fig. 1 with the complex function led to the following constants for the effect of 5A8 alone: $k = 42$, $n = 0.37$, and $m = 0.72$. NEA-9205 alone produced the constants $k = 4.6$, $n = 0.81$, and $m = 0.34$. With these constants the function $f = [c^m/(k + c^m)]^n$ describes the dose-response curves for 5A8 alone and NEA-9205 alone as shown in Fig. 1 and allows the backcalculation of the antibody concentrations required to reach a certain level of inhibition. Let us now calculate the CI for a mixture of 5A8 and NEA9205, in which the concentration of each of the antibodies is equal to 50 ng/ml. The fraction inhibition for that mixture is 0.75. The backcalculated concentrations of the antibodies which alone can achieve that fraction inhibition are 148 ng/ml for 5A8 and 1,100 ng/ml for NEA-9205. Therefore, the formula $c_{1mixt}/c_{1effect} + c_{2mixt}/c_{2effect}$ gives a CI of 50/148 + 50/1,100, or 0.38, which is the value shown in Table 1 for that pair of concentrations.

RESULTS

Synergistic inhibition of HIV-1 envelope glycoprotein-mediated cell fusion by 5A8 in combination with anti-V3 loop antibodies. We have previously found that 5A8, which specifically binds to an epitope of CD4 domain 2, interferes with a post-gp120-binding event that is most likely involved in fusion (3). Numerous investigations have shown that the V3 loop of gp120 plays a major role in post-gp120-binding processes; anti-V3 loop antibodies did not affect binding of gp120 to CD4 but inhibited HIV-1 entry and HIV-1 envelope glycoprotein-mediated cell fusion (for a review, see reference 23). To test the possibility of synergistic inhibition of HIV-1 envelope glycoprotein-mediated membrane fusion by 5A8 and anti-gp120 antibodies, the target CD4 cells (C8166) were preincubated with 5A8 at different concentrations and mixed with chronically HIV-1 IIIB-infected H9 cells which were preincubated with serially diluted anti-V3 loop antibodies (NEA-9205 or 0.5β). Syncytia were counted as a measure of the inhibitory effect. Figure 1 presents the fractions of syncytia which were inhibited with different combinations of 5A8 and NEA-9205. The lines represent fits with empirical formulas which allow calculation of the CI as described in Materials and Methods. The anti-V3 loop antibody NEA-9305 synergized with 5A8, as indicated by the CI, which was less than 1 (Table 1). Similar CIs were found for the other anti-V3 loop MAb, 0.5β (data not shown).

We noted that the inhibitory activities of 5A8, NEA-9205, 0.5β, and other MAbs decline with time. The fraction of syncytia inhibited after 24 h was smaller than that inhibited after 2 h (Fig. 2). Interestingly, however, the synergy between 5A8 and NEA-9205 did not decrease and even increased, as is seen from the lower CIs for syncytium inhibition after 24 h (Table 1 and data not shown).

Similar synergistic effects were observed with another fusion system, where $CD4^-$ 12E1 cells expressing gp120-gp41 encoded by a recombinant vaccinia virus were mixed with $CD4^+$ Molt-3 cells. For this system strong synergy was found between an anti-V3 loop antibody, 110.5, and 5A8 (Fig. 3), with CIs below 0.1 for the entire range of antibody concentrations.

We conclude that anti-V3 loop antibodies in combination

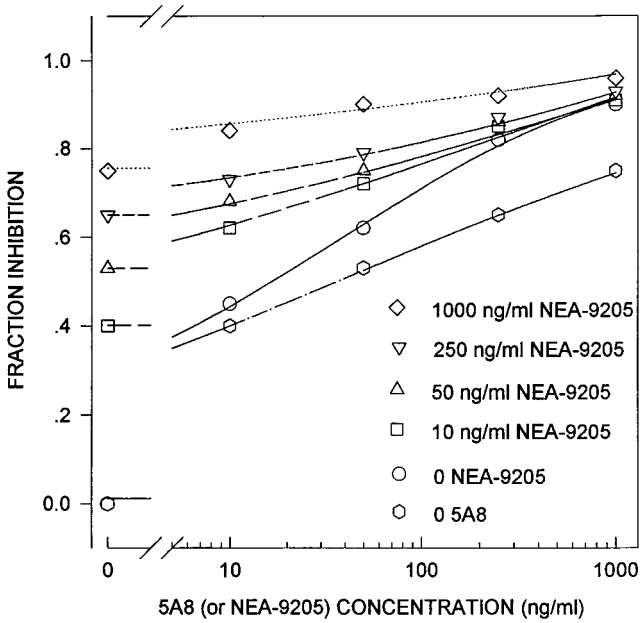

FIG. 1. Synergistic inhibition of fusion by the anti-CD4 MAb 5A8 and the anti-V3 loop MAb NEA-9205. CD4 cells (C8166) were preincubated with different concentrations of 5A8 and mixed with chronically HIV-1 IIIB-infected H9 cells already preincubated with the indicated concentrations of NEA-9205. Syncytia were counted 2 h after mixing. The fraction inhibition was calculated and the experimental points were fitted with empirical formulas as described in Materials and Methods. While most of the curves are plotted to represent the dependence of the fraction of inhibited syncytia as a function of the 5A8 concentration, the bottom curve, designated 0 5A8, represents the dependence of the inhibition as a function of the concentration of NEA-9205.

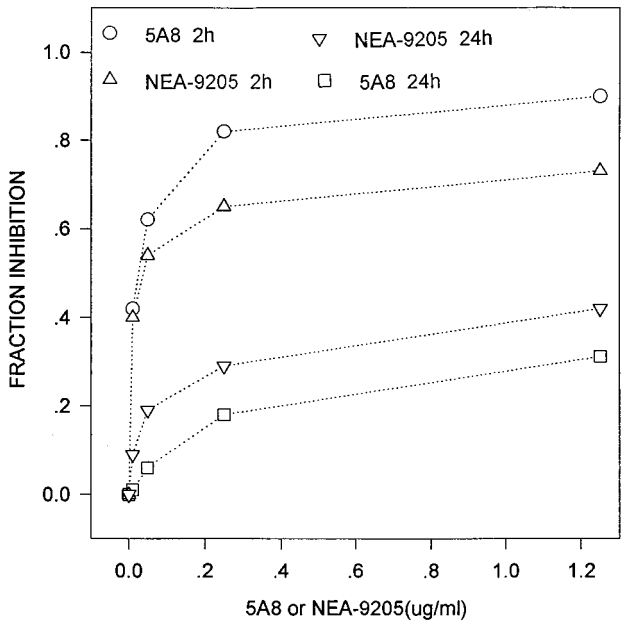

FIG. 2. Fusion inhibition by antibodies decreases with time. CD4 cells (C8166) were preincubated with different concentrations of 5A8 and mixed with chronically HIV-1 IIIB-infected H9 cells. In another set of experiments the chronically infected H9 cells were preincubated with NEA-9205 and mixed with C8166 cells. Syncytia were counted 2 and 24 h after mixing. The fraction of syncytia inhibited by the antibodies was smaller at 24 h than at 2 h for the entire range of concentrations.

with 5A8 synergistically inhibit HIV-1 envelope glycoprotein fusion, and the extent of synergy varies with the antibody epitope and the system used.

Antibodies against the gp120 binding site on CD4 also synergize with anti-V3 loop antibodies. To determine whether the synergistic effect was specific for 5A8, we used two other antibodies against CD4: 6H10, which competes with gp120 for binding on CD4, and OKT4, which does not compete. From the results, shown in Fig. 4, we calculated CIs lower than 1 for 6H10 (Table 2) and of about 1 for OKT4 when used in combination with the anti-V3 loop antibody NEA-9205 (Table 3). Therefore, 6H10 synergizes with NEA-9205, but OKT4, which does not prevent gp120 binding, has only an additive effect, if any. It is interesting that OKT4 has some inhibitory activity at higher concentrations (Fig. 4B). This is probably why the CI is somewhat lower than 1 (Table 3) at those high concentrations and therefore the antibodies seem to synergize, albeit to a very small extent. We conclude from these results that synergy of anti-CD4 antibodies with anti-gp120 antibodies is not specific

TABLE 1. Synergy between the anti-CD4 MAb 5A8 and the anti-V3 loop MAb NEA-9205 in inhibition of syncytium formation between chronically HIV-1 IIIB-infected H9 cells and $CD4^+$ C8166 cells[a]

MAb concn (ng/ml)		CI at:	
5A8	NEA-9205	2 h	24 h
10	10	0.27	0.15
50	50	0.38	0.25
250	250	0.58	0.44
1,000	1,000	0.75	0.46

[a] Cells were preincubated with the antibodies at the indicated concentrations for 30 min at 37°C before mixing. Syncytia were counted 2 and 24 h after mixing. The CI was calculated as described in Materials and Methods.

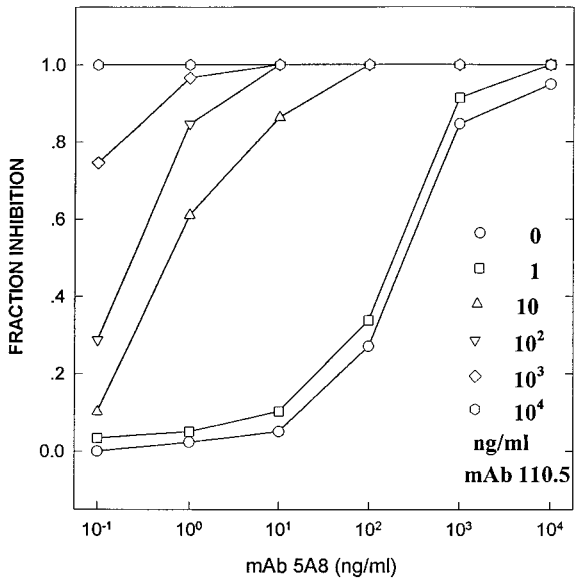

FIG. 3. Synergistic inhibition of fusion by the anti-CD4 MAb 5A8 and the anti-V3 loop MAb 110.5. CD4 cells (Molt-3) were preincubated with different concentrations of 5A8 and mixed with $CD4^-$ 12E1 cells expressing the gp120-gp41 complex encoded by a recombinant vaccinia virus, which were already preincubated with the indicated concentrations of 110.5. Syncytia were counted 3 h after mixing, and the fraction inhibition was calculated as described in Materials and Methods.

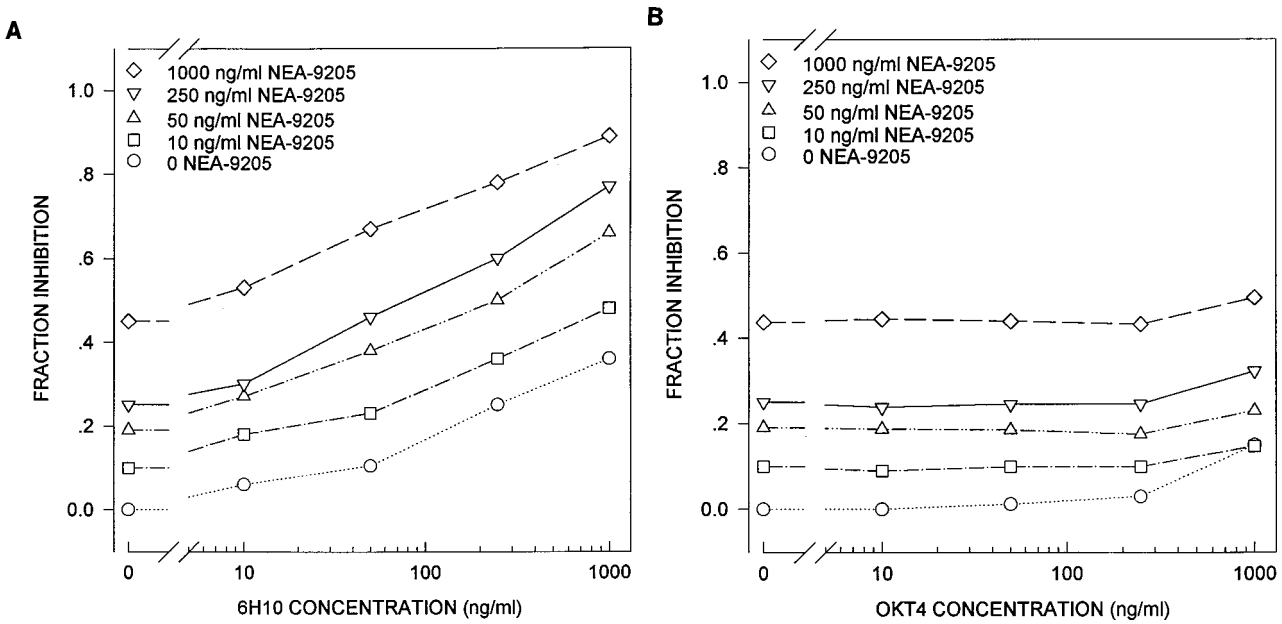

FIG. 4. Synergistic inhibition of fusion by the anti-CD4 MAbs 6H10 (gp120 binding site) and OKT4 (nonneutralizing) combined with the anti-V3 loop MAb NEA-9305. CD4 cells (C8166) were preincubated with different concentrations of 6H10 (A) or OKT4 (B) and mixed with chronically HIV-1 IIIB-infected H9 cells which had been preincubated with the indicated concentrations of NEA-9205. Syncytia were counted 24 h after mixing, and the fraction inhibition was calculated as described in Materials and Methods.

for 5A8 only but also occurs with other anti-CD4 antibodies, particularly antibodies against the gp120 binding site on CD4.

Synergistic fusion inhibitory effect exerted by 5A8 in combination with MAbs to the CD4 binding site of gp120. Recently a human MAb to gp120, IAM 120-1B1, which blocks CD4 binding to gp120 has been developed. This antibody is potentially useful in therapy of HIV-1-infected individuals. To test whether antibodies against the CD4 binding site synergistically inhibit HIV-1 envelope-mediated fusion in combination with 5A8, we preincubated chronically HIV-1 IIIB-infected H9 cells with IAM 120-1B1 and mixed them with C8166 cells preincubated with 5A8. Syncytia were counted 2 h later as a measure of inhibitory effect. We found a synergistic effect on syncytium inhibition (Table 4) which was weaker than that of anti-V3 loop MAbs. With another antibody against the CD4 binding site on gp120, PC39.13, the synergistic effect was stronger and comparable to that of V3 antibodies (Table 5). As with the other antibodies, we noticed that with an increase in the concentrations of the antibodies, the synergistic effect generally decreases and may disappear (Table 4).

Synergistic inhibition of HIV-1 infection and HIV-1 envelope-mediated cell fusion by serum from infected individuals in combination with 5A8. Human serum from HIV-1-infected individuals contains both anti-V3 loop neutralizing antibodies and antibodies to the CD4 binding site of gp120 (22). To test for possible synergy between such serum and 5A8, a patient isolate, 0104B, was preincubated with serum and then used to infect peripheral blood mononuclear cells preincubated with 5A8. HIV-1 infection was synergistically inhibited (Fig. 5) with CIs between 0.2 and 0.5. Interestingly, while 5A8 blocked infectivity (by 75%) at a concentration of 1.25 μg/ml but not (0%) at 125 ng/ml, the combinations with serum diluted 1/800 and 1/1,600, which alone did not affect the infection at all, led to about a 10-fold decrease in the 5A8 concentration required to reach the same level of inhibition (75 to 100%). Similar synergy was observed for cell fusion mediated by the HIV-1 envelope glycoprotein encoded by recombinant vaccinia virus (Fig. 6). As seen in Fig. 6, a 10-fold increase in the 5A8 concentration (from 10 to 100 ng/ml) leads to only about a 10% increase in inhibition. However, in the presence of serum

TABLE 2. Synergy between the anti-CD4 (gp120 binding site) MAb 6H10 and the anti-V3 loop MAb NEA-9205 in inhibition of syncytium formation between chronically HIV-1 IIIB-infected H9 cells and $CD4^+$ C8166 cells[a]

MAb concn (ng/ml)		CI
6H10	NEA-9205	
10	10	0.23
50	50	0.23
250	250	0.33
1,000	1,000	0.18

[a] Cells were preincubated with the antibodies at the indicated concentrations for 30 min at 37°C before mixing. Syncytia were counted 24 h after mixing. The CI was calculated as described in Materials and Methods.

TABLE 3. Lack of synergy between the control anti-CD4 MAb OKT4 and the anti-V3 loop MAb NEA-9205 in inhibition of syncytium formation between chronically HIV-1 IIIB-infected H9 cells and $CD4^+$ C8166 cells[a]

MAb concn (ng/ml)		CI
OKT4	NEA-9205	
10	10	0.96
50	50	1.12
250	250	1.04
1,000	1,000	0.89

[a] Cells were preincubated with the antibodies at the indicated concentrations for 30 min at 37°C before mixing. Syncytia were counted 24 h after mixing. The CI was calculated as described in Materials and Methods.

TABLE 4. Synergy between the anti-gp120 (CD4 binding site) MAb IAM 120-1B1 and the anti-CD4 MAb 5A8 in inhibition of syncytium formation between chronically HIV-1 IIIB-infected H9 cells and $CD4^+$ C8166 cells[a]

MAb concn (ng/ml)		CI
5A8	IAM 120-1B1	
3	50	0.43
3	250	0.85
12	50	0.77
12	250	0.95

[a] Cells were preincubated with the antibodies at the indicated concentrations for 30 min at 37°C before mixing. Syncytia were counted 2 h after mixing. The CI was calculated as described in Materials and Methods.

(at a dilution of $1/10^4$), which alone resulted in about a 20% inhibition, the inhibition of cell fusion was about 70% at 10 ng/ml of 5A8. We conclude that with 5A8, patient serum can inhibit HIV-1 infection and cell fusion synergistically, and even very small concentrations of 5A8 are efficient when combined with highly diluted patient serum.

DISCUSSION

This study demonstrates that antibodies directed to the HIV-1 envelope glycoprotein and an antibody to the CD4 second domain, 5A8, can synergistically inhibit gp120-gp41-mediated membrane fusion and HIV-1 infection. The synergistic effect was also exhibited when the anti-CD4 antibody was combined with human serum from HIV-1-infected individuals, which presumably contains both anti-V3 loop and anti-CD4 binding site antibodies. These findings are interesting in light of the recently reported lack of significant cell loss or immunosuppression in rhesus monkeys after their $CD4^+$ lymphocytes were coated with 5A8 for >9 days (26). Our results suggest that if 5A8 is used in combination with anti-gp120 antibodies, its therapeutic concentration can be decreased, which will further decrease possible immunosuppressive effects.

Especially important is the observation that patient serum also has a potent synergizing effect. This suggests that patients who already have anti-gp120 antibodies, whose efficiency is not sufficient to block the infection, especially in the late stages of the HIV-1 disease, may experience a powerful potentiating effect with 5A8. Moreover, since the 5A8 epitope is not dependent on the particular HIV-1 isolate, the 5A8 inhibitory effect may affect escape mutants as well as it does the dominant virus strain. Therefore, the synergistic effects of anti-CD4 antibodies and anti-gp120 antibodies can provide a new approach to the immunotherapy and prevention of AIDS. One must emphasize, however, that these results were obtained in tissue cultures with only two samples of patient serum and several MAbs. Their significance for the in vivo system requires further experimentation.

TABLE 5. Synergy between the anti-gp120 (CD4 binding site) MAb PC39.13 and the anti-CD4 MAb 5A8 in inhibition of syncytium formation between chronically HIV-1 IIIB-infected H9 cells and $CD4^+$ C8166 cells[a]

5A8 concn (ng/ml)	PC39.13 dilution	CI
3	1/1,600	0.24
125	1/1,600	0.26
25	1/400	0.42
125	1/400	0.40

[a] Cells were preincubated with the antibodies at the indicated concentrations for 30 min at 37°C before mixing. Syncytia were counted 24 h after mixing. The CI was calculated as described in Materials and Methods.

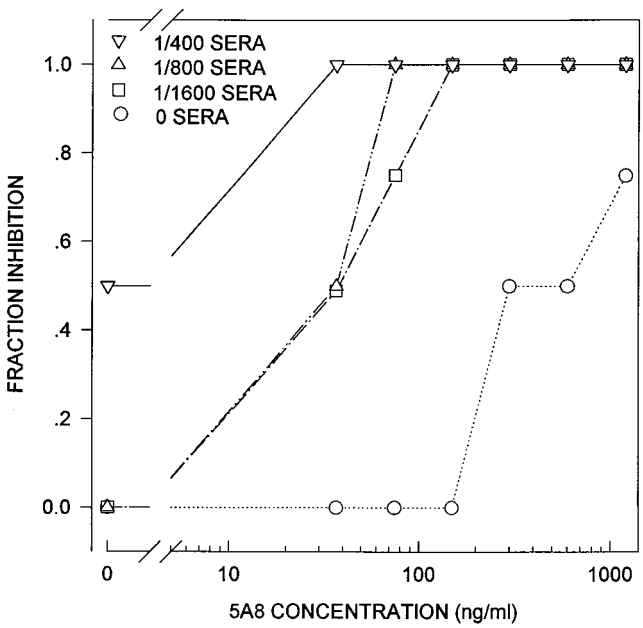

FIG. 5. Synergistic neutralization of the HIV-1 patient isolate (0104B) by the anti-CD4 MAb and patient serum. The phytohemagglutinin-treated peripheral blood mononuclear cells were incubated with 5A8 for 30 min at 37°C and then mixed with the virus, which had been preincubated with patient serum for 30 min. The virus-cell mixture was incubated overnight and then washed and cultured. The MAb concentration was maintained throughout the culture, while the patient serum was added only in the beginning of the cell culture. The results are the averages for two independent experiments, each with duplicated culture wells. The infected cultures were counted after 2 weeks, and the fraction inhibition was calculated as described in Materials and Methods.

The mechanism underlying the synergistic effects is not known. It has been shown that soluble CD4 (sCD4) or a MAb, 39.13g, binding to a conformational epitope of gp120 involved in CD4 binding synergistically neutralizes HIV-1 infection when combined with MAbs binding to the V3 domain of gp120 (20). Interestingly, the MAbs to the V3 loop showed increased binding to virion gp120 in the presence of sCD4, suggesting a possible mechanism for the synergistic neutralization (20). Several other studies have also shown that sCD4 (25) and anti-CD4 binding site MAbs synergize with HIV-1-neutralizing anti-V3 loop MAbs (2, 17, 24, 27). However, the effects of sCD4 and anti-HIV-1 human serum were found to be additive or only slightly synergistic (15).

Our studies now show that synergy can also be achieved with the 6H10 MAb directed against the gp120 binding site on CD4 and anti-V3 loop MAbs. Thus, agents which interfere with the gp120-CD4 binding step generally synergize with anti-V3 loop MAbs. The use of agents which target molecules associated with different membrane surfaces may indicate that the cooperativity of MAb binding is not required to achieve this synergy. In addition, we have now shown that anti-gp120 MAbs which block CD4 binding can synergize with the 5A8 MAb, which blocks a post-gp120 binding step, albeit to a lesser extent than the anti-V3 loop MAbs synergize with 5A8. Interestingly, we observed that while the inhibitory activity of the antibodies we tested (including 5A8 and NEA-9205) decreases with time, probably because of the production of new envelope glyco-

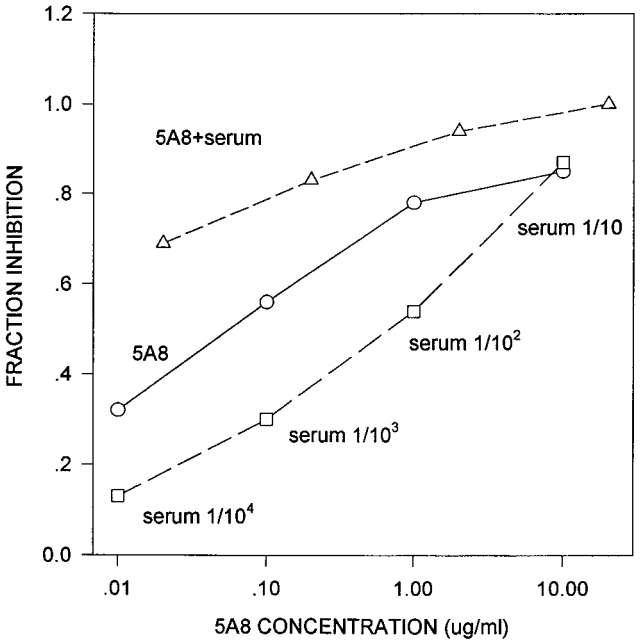

FIG. 6. Synergistic inhibition of cell fusion by the anti-CD4 MAb 5A8 and patient serum. CD4 cells (Molt-3) were preincubated with different concentrations of 5A8 and mixed with $CD4^-$ 12E1 cells expressing the gp120-gp41 complex encoded by a recombinant vaccinia virus which had already been preincubated with the indicated dilutions of the serum. The dilutions of serum in the mixture are the same as those given for the bottom curve. The points for the mixture are shifted to the right to indicate that the total antibody concentration is higher than the 5A8 concentration. Syncytia were counted 24 h after mixing, and the fraction inhibition was calculated as described in Materials and Methods.

protein molecules, the synergy between the antibodies did not significantly change. How this is related to the mechanism of synergy is unclear at this point.

The CIs which we calculated for inhibition of fusion between HIV-1-infected cells and $CD4^+$ cells are in the same range (0.2 to 0.95) as those reported in most of the recent studies (2, 11, 24, 27) except that of McKeating et al. (20), in which some of the values are below 0.01. As also observed by other groups, the CIs varied with the epitope and the concentration of the antibodies. Interestingly, we observed the highest synergy at low antibody concentrations. This may imply that the synergy is due to the disruption of fusion complexes, which requires the cooperation of more than one molecule of the envelope glycoprotein and its receptor. Such cooperation has recently been suggested on the basis of data for dominant interference of a fusion-deficient mutant (11, 12). One might speculate that inactivation of one molecule from the fusion complex leads to the inactivation of the entire complex. At low concentrations the anti-CD4 and anti-gp120 antibodies may inactivate more complexes in combination than alone at the same concentration. At a high concentration the surface-bound antibody is close to saturation, and a further increase in concentration may not lead to an increase in inhibition. While this mechanism is just a speculation, the very fact that the CIs in most of the studies are in the same range indicates that the underlying mechanism may be the same and therefore not significantly dependent on the particular interaction leading to neutralization. Future studies are needed to elucidate how antibodies synergize in neutralizing HIV-1 envelope glycoprotein-mediated membrane fusion.

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