The B-Oligomer of Pertussis Toxin Deactivates CC Chemokine Receptor 5 and Blocks Entry of M-tropic HIV-1 Strains

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Summary

Infection of target cells by HIV-1 requires initial binding interactions between the viral envelope glycoprotein gp120, the cell surface protein CD4, and one of the members of the seven-transmembrane G protein–coupled chemokine receptor family. Most primary isolates (R5 strains) use chemokine receptor CCR5, but some primary syncytium-inducing, as well as T cell line–adapted, strains (X4 strains) use the CXCR4 receptor. Signaling from both CCR5 and CXCR4 is mediated by pertussis toxin (PTX)-sensitive G_i proteins and is not required for HIV-1 entry. Here, we show that the PTX holotoxin as well as its binding subunit, B-oligomer, which lacks G_i-inhibitory activity, blocked entry of R5 but not X4 strains into primary T lymphocytes. Interestingly, B-oligomer inhibited virus production by peripheral blood mononuclear cell cultures infected with either R5 or X4 strains, indicating that it can affect HIV-1 replication at both entry and post-entry levels. T cells treated with B-oligomer did not initiate signal transduction in response to macrophage inflammatory protein (MIP)-1β or RANTES (regulated upon activation, normal T cell expressed and secreted); however, cell surface expression of CCR5 and binding of MIP-1β or HIV-1 to such cells were not impaired. The inhibitory effect of B-oligomer on signaling from CCR5 and on entry of R5 HIV-1 strains was reversed by protein kinase C (PKC) inhibitors, indicating that B-oligomer activity is mediated by signaling events that involve PKC. B-oligomer also blocked cocapping of CCR5 and CD4 induced by R5 HIV-1 in primary T cells, but did not affect cocapping of CXCR4 and CD4 after inoculation of the cultures with X4 HIV-1. These results suggest that the B-oligomer of PTX cross-deactivates CCR5 to impair its function as a coreceptor for HIV-1.

Key words: CCR5 • signal transduction • G_i protein • receptor capping • receptor desensitization

Infection of the target cells by HIV-1 is initiated by interaction between the viral envelope protein, gp120, and a specific set of cell surface receptors. In addition to CD4, which has long been recognized as an essential component of the receptor for HIV and SIV (1), several chemokine receptors have been shown recently to function as coreceptors (for review see reference 2). Despite a wide variety of chemokine receptors, all primary M-tropic strains of HIV-1 described to date have been shown to be capable of using CC chemokine receptor (CCR)5[1] (3–8), a receptor for CC chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (regulated upon activation, normal T cell expressed and secreted). The major coreceptor for T cell line–adapted HIV-1 strains is CXCR4 (9), a receptor for a CXC chemokine, stroma-derived factor (SDF)-1α. CXCR4 can be used also by syncytium–inducing primary strains that appear at the late stages of AIDS progression (8, 10–12).

Chemokine receptors belong to a group of seven-transmembrane receptors that transduce signals via coupling to G proteins. Both CCR5 and CXCR4 are believed to be coupled to G_i-like proteins, based on their sensitivity to pertussis toxin (PTX) (13). Binding of a ligand (a chemokine or HIV-1) to these receptors induces a characteristic Ca^{2+} flux and tyrosine phosphorylation (13–15), which can be blocked by pretreatment of the cells with PTX. However, this signaling appears to be unimportant for the func-

[1]*Abbreviations used in this paper:* CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PKC, protein kinase C; PTX, pertussis toxin; RANTES, regulated upon activation, normal T cell expressed and secreted; SDF, stroma-derived factor.

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tion of chemokine receptors as coreceptors for HIV-1, at least in immortalized cells overexpressing chemokine receptors (16–20). Indeed, transfection into CCR5-negative cells of mutant receptors unable to couple to G proteins and transduce signals makes such cells fully susceptible to infection with R5 HIV-1 strains. In contrast, HIV-1 infection of primary $CD4^+$ T cells appears to require actin-mediated rearrangement of receptors (21), implying a signal-mediated process.

PTX is the major virulence factor of *Bordetella pertussis*, the causative agent of whooping cough. PTX is a 105-kD noncovalently linked heterohexameric protein, which can be functionally divided into an enzymatically active A-protomer and a B (binding)-oligomer. The A-protomer consists of a single peptide subunit (S1) with ADP-ribosyltransferase activity, which specifically ribosylates and inactivates the α-subunit of G_i proteins, thus leading to uncoupling of corresponding signal transduction events (22, 23). The B-oligomer is a pentameric protein complex composed of two dimers (S2-S4 and S3-S4) joined together by the S5 subunit, and is responsible for target cell binding (for review see reference 24). The preferential binding sites for PTX are carbohydrate moieties (25), but cell surface molecules bearing these carbohydrate determinants have not yet been unequivocally identified. In lymphocytes, a 70-kD protein (p70) exhibiting features of the PTX receptor has been described (26–28); however, p70 may be only one part of a complex receptor, as PTX was shown to interact also with smaller cell surface proteins of 43 and 50 kD (27, 29). Treatment of T lymphocytes with PTX or purified B-oligomer induced a signaling response typical of ligand–receptor interaction, characterized by an increase of diacylglycerol levels and protein kinase C (PKC) activity, and by Ca^{2+} flux (30–32). Thus, it is not surprising that a number of biological effects of PTX are mediated by its B-oligomer, independently of G_i protein inactivation (for review see reference 24).

One such activity of PTX and B-oligomer is described in this report. We demonstrate that treatment of primary T cells with PTX or B-oligomer induces a specific desensitization of CCR5. As a result, such cells do not respond to stimulation with a CCR5 ligand, MIP-1β, and do not support entry of CCR5-dependent HIV-1 strains.

Materials and Methods

Reagents. PTX was purchased from Sigma Chemical Co. and B-oligomer was from Calbiochem. The purity of B-oligomer was verified by gel electrophoresis analysis, which revealed complete lack of contamination with A-protomer. MIP-1β, RANTES, and SDF-1α were from PeproTech, and Fura-2/AM, Ro-31-8220, and Gö 6979 were from Calbiochem.

Primary Lymphocyte Cultures. T cell–enriched, monocyte-depleted cultures were established from PBMCs from HIV-1–negative donors by Ficoll-Hypaque gradient centrifugation and two rounds of adherence to plastic. Nonadherent cells were collected by centrifugation, resuspended in RPMI 1640 supplemented with 10% heat-inactivated FCS, and stimulated with phytohemagglutinin (5 μg/ml) for 3 d. Cells were then washed and cultured for another 7 d in medium supplemented with 20 U/ml of recombinant IL-2. By that time, ~84% of the cells were $CD3^+$, ~40% were $CD4^+$, ~30% were $CCR5^+$, ~95% were $CXCR4^+$, ~10% were $CCR5^+CD4^+$, and ~35% were $CXCR4^+CD4^+$, as determined by flow cytometry.

Infection with HIV-1. Five HIV-1 strains were used in this study: three R5 viruses (92US660, 92US657, and ADA) and two X4 strains (92UG21, a primary isolate, and LAI, a T cell line–adapted virus). Before infection, viral stocks were treated with 200 U/ml of RNase-free DNase for 1 h at room temperature to eliminate DNA contamination. Viral inoculae were adjusted according to reverse transcriptase activity to 6×10^4 cpm per 10^6 cells. After a 2-h adsorption, cells were washed and cultured in IL-2–supplemented medium.

Immunofluorescent Microscopy. Cells were treated with B-oligomer (1 nM) for 10 min and then inoculated with heat-inactivated HIV-1 (2×10^6 cpm/ml of reverse transcriptase activity) or with 5 μg/ml of recombinant $gp120_{JR\text{-}FL}$ (Progenics Pharmaceuticals) or $gp120_{LAI}$ (Intracel). After 60 min at 37°C, cells were washed and fixed in 4% buffered formaldehyde. After washing, cells were incubated for 20 min in 1% FCS/PBS at room temperature, and 2×10^5 cells were incubated for 30 min at room temperature in 0.1 ml of 1% FCS/PBS with anti-CCR5 or anti-CXCR4 mAb (2D7 and 12G5, respectively, obtained from PharMingen; both antibodies neutralize HIV-1 infection), followed by a 30-min incubation with a secondary rhodamine-labeled anti–mouse IgG. After washing, cells were incubated with FITC-labeled anti-CD4 mAb (13B8.2, Immunotech; this antibody does not compete with gp120 for CD4 binding), then washed, spotted on slides, dried, and analyzed on an immunofluorescent imaging system using a dedicated software (MetaMorph; Universal Imaging Corporation).

Binding Studies. For each binding reaction, 10^6 cells resuspended in binding buffer (50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% BSA) were mixed with B-oligomer (1 nM), incubated for 10 min at 37°C, and then transferred to ice. 550 pM of ^{125}I-labeled MIP-1β (specific activity 2,200 Ci/mmol) was added to cells in the presence or absence of a 1,000-fold molar excess of unlabeled MIP-1β, or 0.5 μg/ml of gp120, and reactions were incubated at 4°C for 4 h on a horizontal shaker. After washing with binding buffer containing 0.5 M NaCl, cell-bound radioactivity was counted in a gamma-counter.

Calcium Mobilization Assay. The assay was performed as previously described (33). In brief, 0.6 ml of Fura-2–loaded cells (5×10^6 cells/ml) was transferred to an acrylic cuvette and stimulated with B-oligomer (500 ng/sample), SDF-1α (100 ng/sample), or MIP-1β (500 ng/sample). Fluorescence emission at 340 and 380 nm was measured on a Perkin-Elmer Luminescence Spectrometer LS50B.

Results and Discussion

Several previously published reports (16–20) demonstrated that activity of chemokine receptors as coreceptors for HIV-1 does not involve signaling via coupled G_i proteins. A somewhat conflicting result has been reported recently (21), demonstrating that entry of X4 HIV-1 strains into primary T cells correlated with actin-dependent cocapping of CD4 and CXCR4 receptors, thus implicating signaling in the process of HIV-1 entry. To better define the role of signaling from chemokine receptors in HIV-1

entry into primary T cells, we used PTX to specifically inactivate G_i-like proteins that transduce signals from both CCR5 and CXCR4 (13), and measured HIV-1 entry by PCR, using primers LTR R/U5 specific for early products of reverse transcription (34). The fragment of HIV-1 cDNA amplified by these primers is produced either within the virion or very early after virus–cell fusion, and thus reflects the efficiency of virus entry. Surprisingly, PTX inhibited entry of R5 HIV-1 strains 92US660 (Fig. 1 A) and ADA (not shown), but not of X4 strains LAI (Fig. 1 A) and 92UG21 (not shown). PTX is a complex protein composed of an active (A) and a binding (B) subunit, and certain T cell activities have been shown to be initiated by the B-oligomer of PTX independently of inactivation of G_i-like proteins by the A-protomer (35). We therefore tested whether activity of B-oligomer could account for the observed inhibitory effect of PTX on entry of R5 HIV-1. Similar to results observed with PTX, B-oligomer inhibited entry of R5 HIV-1 strains in a dose-dependent fashion, but not of X4 HIV-1 strains (Fig. 1 B). Analysis of the B-oligomer preparation by SDS-PAGE confirmed the lack of A-protomer (not shown), and G_i-mediated signaling was not impaired in B-oligomer–treated cells (see Fig. 4, right). We thus conclude that PTX blocks entry of R5 HIV-1 strains by a mechanism that is independent of G_i protein inhibition. Interestingly, no inhibitory activity of PTX or B-oligomer was observed on entry of R5 HIV-1 into PM1 cells (not shown), consistent with recently reported results (36). This result underscores the differences between the primary cells and T cell lines.

To analyze the effect of B-oligomer on HIV-1 replication in long-term cultures, we measured virus production after in vitro infection of primary PBMCs from HIV-seronegative donor with R5 or X4 HIV-1 strains. As predicted from the entry studies (Fig. 1), B-oligomer at 1 nM concentration inhibited replication of a primary R5 strain, 92US660 (Fig. 2 A). Unexpectedly, a similar level of inhibition was also observed with primary (92UG21; Fig. 2 B) and cell line–adapted (LAI; Fig. 2 C) X4 strains, entry of which was not affected by B-oligomer (Fig. 1). This result is consistent with recently reported (37) inhibitory effect of PTX on replication in PBMCs of another X4 strain, RF, and indicates that, in addition to its effect on HIV-1 entry via the CCR5 receptor, B-oligomer exerts its anti-HIV activity via another, as yet unidentified, mechanism that works at the post-entry step of viral replication and does not depend on the identity of chemokine receptor used by the virus. Interestingly, this post-entry inhibitory effect of B-oligomer depended on the multiplicity of infection and was overcome when high virus inoculum was used (data not shown). Similar to results of entry studies, replication of neither R5 (92US660) nor X4 (LAI) strains was inhibited in PM1 cells (Fig. 2, A and C, insets). To further investigate the anti-HIV activity of B-oligomer, we analyzed its effect on replication of uncloned primary HIV-1 in cocultures of uninfected donor PBMCs with activated PBMCs from HIV-1–infected patients. The number of infected cells in the PBMCs from such patients is usually low (38), so the measured virus output in cocultures is primarily a result of virus spread to new targets. A dose-dependent inhibition of virus replication by B-oligomer was observed (Fig. 2 D). This effect was reproduced with PBMCs from three different patients (not shown); in all cases 1 nM of B-oligomer completely inhibited virus replication. To demonstrate that the inhibitory effect of B-oligomer was not due to its cytotoxic activity, we measured uptake of [^{3}H]thymidine and the change in cell numbers in long-term cultures. As shown in Fig. 2 E, no significant difference in thymidine uptake between B-oligomer–treated and untreated cultures was observed during the whole course of

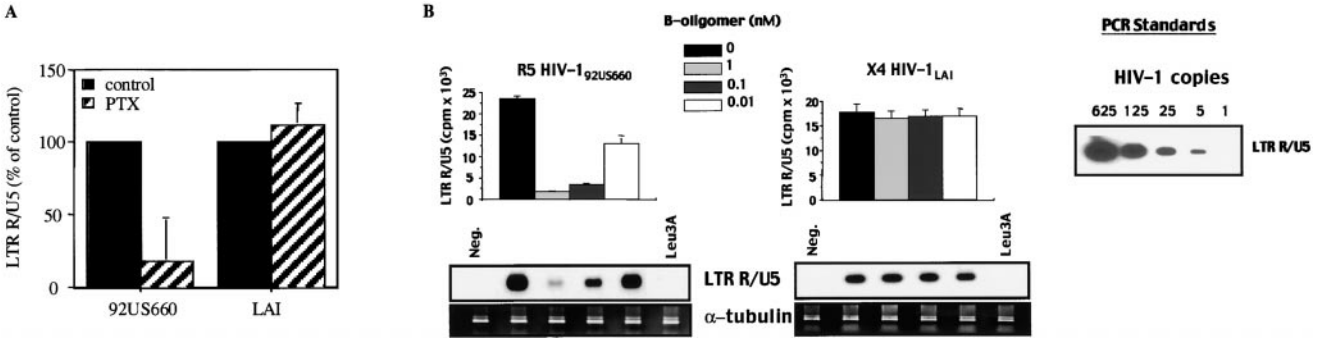

Figure 1. The effect of PTX and B-oligomer on HIV-1 entry. (A) Monocyte-depleted PBMC cultures were treated with PTX (1 nM) or left untreated (control), and inoculated with R5 HIV-$1_{92US660}$ or X4 HIV-1_{LAI}. Viral entry was analyzed by PCR using primers LTR R/U5 specific for the early reverse transcription product, as described previously (34). Results were quantified on a Direct Imager (Packard Instrument Company) and are presented as a percentage of counts in treated versus control samples. The error bars show the deviation from the mean for triplicate samples. Similar results were obtained in two independent experiments with cells from different donors. (B) Cells were treated with the indicated concentrations of B-oligomer or an anti-CD4 mAb Leu3A for 10 min or were left untreated and inoculated with HIV-$1_{92US660}$ (left) or HIV-1_{LAI} (middle). Viral entry was analyzed as in A. In parallel, each sample was amplified with α-tubulin–specific primers to control for the total amount of DNA. Results were quantified on a Packard Direct Imager and are presented on bar graphs as mean counts for each set of duplicate samples, expressed as counts per minute (cpm). The error bars show the deviation from the mean for each duplicate. Uninfected cells (Neg.) served as negative control. Dilutions of 8E5/LAI cells containing one HIV-1 genome per cell (45) were used as PCR standards (right).

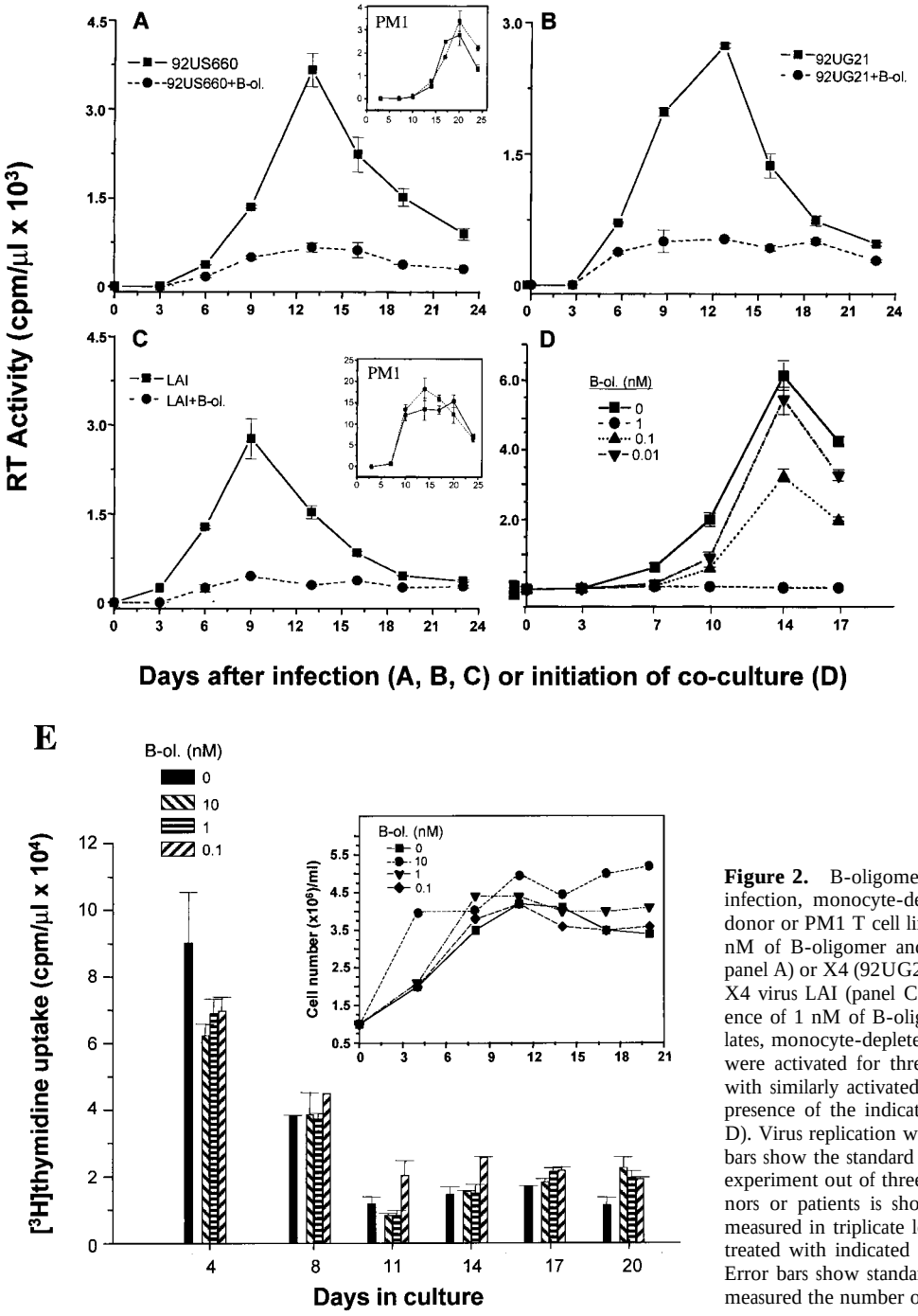

Figure 2. B-oligomer inhibits HIV-1 replication. For in vitro infection, monocyte-depleted PBMCs from HIV seronegative donor or PM1 T cell line (insets) were pretreated for 1 h with 1 nM of B-oligomer and infected with primary R5 (92US660, panel A) or X4 (92UG21, panel B) strains, or T cell line–adapted X4 virus LAI (panel C). Subsequent culturing was in the presence of 1 nM of B-oligomer. For direct analysis of clinical isolates, monocyte-depleted PBMCs from HIV-1–infected patients were activated for three days with PHA and then cocultured with similarly activated PBMCs from uninfected donors in the presence of the indicated concentrations of B-oligomer (panel D). Virus replication was assayed in triplicate cultures, and error bars show the standard deviations of the mean. A representative experiment out of three performed with cells from different donors or patients is shown. The uptake of [^{3}H]thymidine was measured in triplicate long-term cultures of uninfected PBMCs treated with indicated concentrations of B-oligomer (panel E). Error bars show standard deviation of the mean. In parallel we measured the number of cells in each culture (Fig. 2 E, inset).

the experiment. In fact, treated cultures demonstrated higher proliferation rate (Fig. 2 E, inset), consistent with the well-known mitogenic effect of B-oligomer (24). Therefore, B-oligomer inhibits replication of a wide variety of primary and cell line–adapted isolates. This result may explain a recently reported inhibitory effect of PTX on replication of SIV_{mac251} (which uses the CCR5 receptor) in vivo (39). The inhibitory effect of B-oligomer is exerted at both the entry (for R5 strains) and post-entry (probably for both R5 and X4 viruses) steps of HIV-1 infection. In this work, we concentrated on the mechanisms of B-oligomer–mediated inactivation of CCR5.

A possible mechanism by which B-oligomer could specifically inhibit entry of R5 but not X4 HIV-1 strains is by interfering with binding of the virus, either directly (by blocking the HIV-1 binding site on CCR5) or indirectly (by changing conformation of CCR5). To test this possibility, we performed binding studies with ^{125}I-labeled MIP-1β and gp120. Results in Fig. 3 A demonstrate that binding of MIP-1β to PBMCs was competed by recombinant

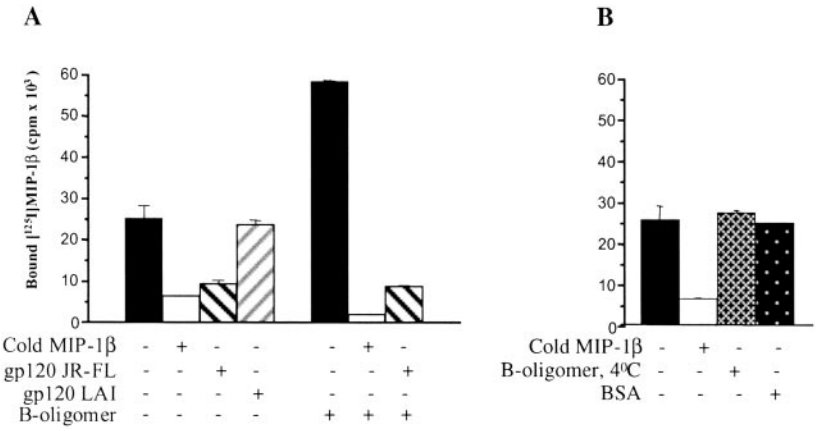

Figure 3. B-oligomer does not block ligand binding to CCR5. Analysis of ^{125}I-labeled MIP-1β binding to untreated or B-oligomer–treated PBMCs was performed as described in Materials and Methods. Binding was also measured in the presence of either a 1,000-fold molar excess of unlabeled (cold) MIP-1β, or 0.5 μg/ml of gp120 of an R5 (JR-FL) or X4 (LAI) HIV-1 strain (A). To control for nonspecific effects of the B-oligomer, binding was analyzed also using cells treated either with B-oligomer (1 nM) at 4°C, or with 75 ng/ml of BSA at 37°C (B). Results are presented as means of duplicate samples, and the error bars show the deviation from the mean for each duplicate.

gp120 of an R5 strain, HIV-1_{JR-FL}, but not by gp120 of an X4 strain, HIV-1_{LAI}. Although treatment of the cells with B-oligomer increased ^{125}I-labeled MIP-1β binding threefold, binding of this ligand was still effectively competed by $gp120_{JR-FL}$ (Fig. 3 A). To exclude the possibility that B-oligomer nonspecifically affected binding of CCR5 ligands, for instance by acting as a bridge between the ligand and the cell, analysis was also performed using cells treated for 10 min either with B-oligomer at 4°C (to block signaling from B-oligomer receptor), or with BSA at 37°C (to mimic the protein concentration in the experimental samples). Both treatments had no effect on ^{125}I-labeled MIP-1β binding, compared with untreated sample (Fig. 3 B). Therefore, we conclude that the inhibitory activity of the B-oligomer on HIV-1 entry is a signal-mediated process that does not involve blocking of HIV-1 binding to its receptors. This result also suggests that binding of gp120 to CD4 and CCR5 is not sufficient to induce fusion of the viral and cellular membranes.

A logical conclusion from the results presented above is that a post-binding step of virus entry was inhibited in B-oligomer–treated cells, and that this effect could be mediated by B-oligomer–induced alteration of an intracellular portion of the CCR5 molecule. As this part of the receptor is involved in interactions with coupled G proteins, we measured Ca^{2+} mobilization in response to natural ligands of CCR5 and CXCR4, MIP-1β or RANTES, and SDF-1α, respectively, after pretreatment of the cells with B-oligomer. Treatment of the cells with B-oligomer blocked Ca^{2+} flux initiated by MIP-1β (Fig. 4 A) and RANTES (not shown), but not by SDF-1α (Fig. 4 B). Although pretreatment of the cells with MIP-1β desensitized response to B-oligomer (Fig. 4 C), pretreatment with SDF-1α induced only partial desensitization (Fig. 4 D). Of note, B-oligomer at the suboptimal concentration used in these experiments (2 nM) did not desensitize its own receptor (not shown). The use of higher concentrations of B-oligomer for desensitization studies was confounded by a very slow return of the Ca^{2+} response to the baseline (>30 min), thus precluding analysis by Fura-2–based technique due to leaking of the dye from the cells. The pattern of Ca^{2+} response to sequential treatment of cells with B-oligomer and MIP-1β is best described by the phenomenon of heterologous desensitization. Potentially, this desensitization is attributable to signaling from a B-oligomer receptor, similar to the heterologous desensitization of CXCR1 and CXCR2 chemokine receptors by signaling from opiate receptors (40).

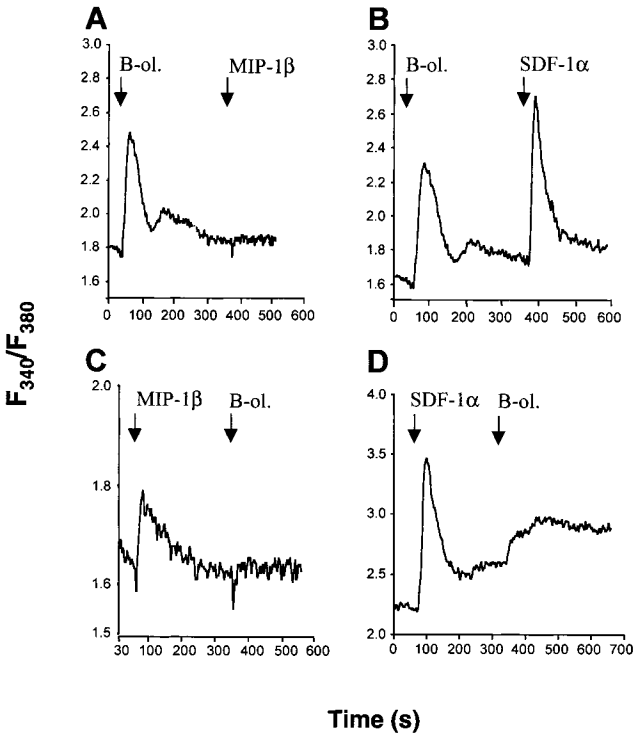

Figure 4. Analysis of cross-desensitization of chemokine and PTX receptors. Fura-2–loaded PBMCs were stimulated with 0.83 μg/ml of B-oligomer (B-ol.) (A and B), 1.66 μg/ml of MIP-1β (C), or 0.1 μg/ml of SDF-1α (D) at the time indicated by the left arrow. At the time indicated by the right arrow (3–5 min later), cells were rechallenged with B-oligomer (C and D), MIP-1β (A), or SDF-1α (B) at the same concentration as in the first challenge. Results of one representative experiment out of three performed with cells from different donors are presented as the ratio of fluorescence emissions at 340 and 380 nm (F_{340}/F_{380}) over time.

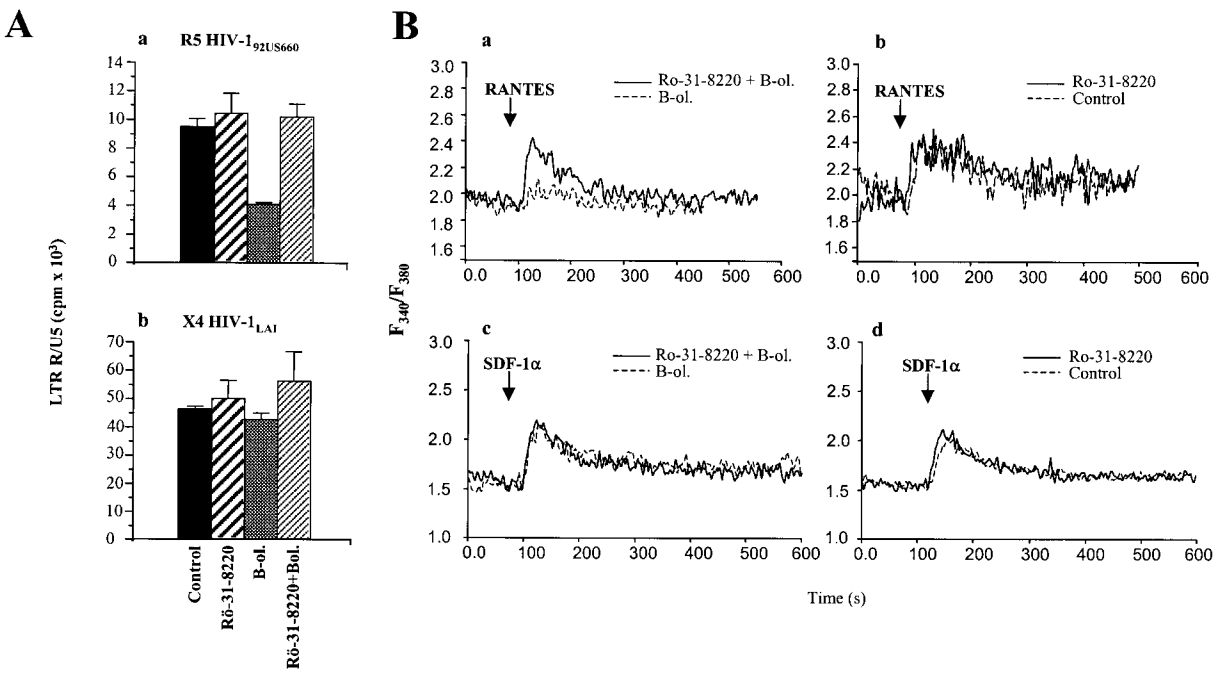

Figure 5. The effect of B-oligomer on CCR5 is reversed by PKC inhibitor. (A) Ro-31-8220 reverses the inhibitory effect of B-oligomer on entry of R5 strains. PBMC cultures were either left untreated (control) or treated with B-oligomer (1 nM for 10 min) with or without Ro-31-8220 (pretreatment for 20 min with 10 nM of the inhibitor). Entry of R5 (92US660) or X4 (LAI) strains of HIV-1 was measured as described in the legend to Fig. 1. Results are presented as mean of two independent experiments with cells from the same donor. The error bars show the deviation from the mean for duplicate samples. Similar results were obtained with cells from a different donor. (B) Ro-31-8220 reverses the inhibitory effect of B-oligomer on signaling from CCR5. PBMC cultures were either treated with B-oligomer (1 nM) alone or in combination with Ro-31-8220 (10 nM) (panels a and c) or left untreated (control) or treated with Ro-31-8220 (100 nM) alone (panels b and d) and stimulated with 500 ng/ml of RANTES (panels a and b) or 100 ng/ml of SDF-1α (panels c and d) at the time indicated by the arrow. Ca^{2+} flux was measured as described in the legend to Fig. 4.

Although we can not formally rule out the possibility that B-oligomer binds to CCR5, several considerations make this scenario unlikely. First, B-oligomer did not compete with MIP-1β or gp120 for binding to CCR5 (Fig. 3). Second, B-oligomer did not induce downregulation of CCR5, as evidenced by flow cytometric analysis (not shown). Third, B-oligomer did not induce Ca^{2+} flux (not shown) and did not affect HIV-1 replication (Fig. 2) in PM1 cells, support-

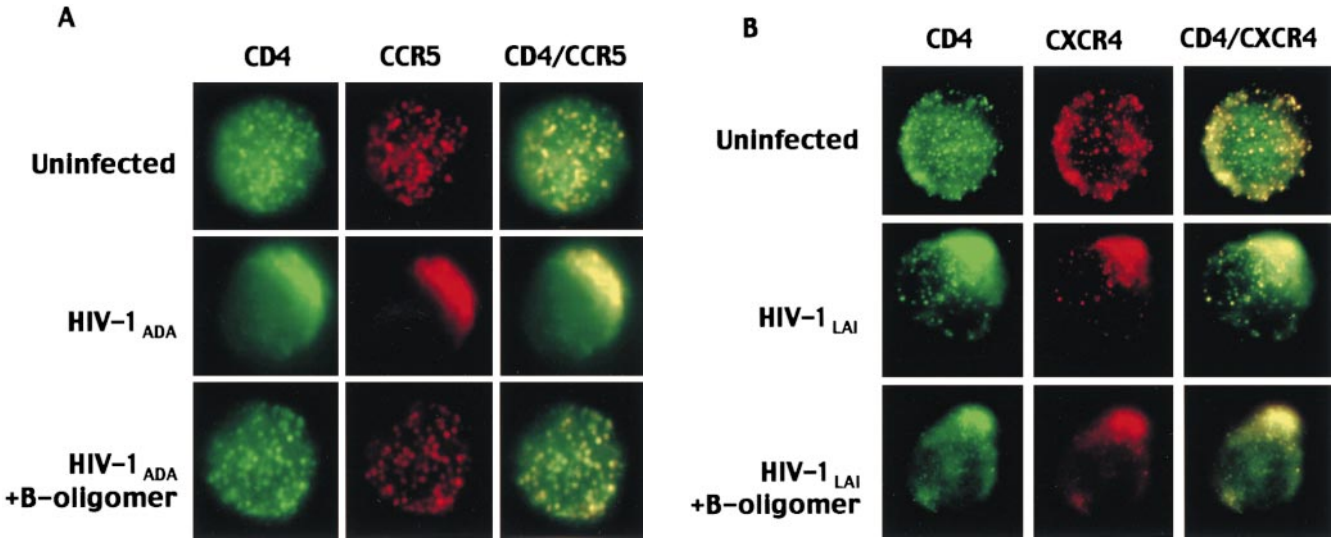

Figure 6. Analysis of receptor capping by immunofluorescent microscopy. PBMCs were treated with 1 nM of B-oligomer for 10 min or left untreated, and receptor capping was analyzed by a dual-color immunofluorescent microscopy after inoculation with R5 HIV-$1_{92US660}$ (A) or X4 HIV-1_{LAI} (B) viruses. CD4 was revealed with FITC-conjugated anti-CD4 mAb (thus producing green fluorescence), while chemokine receptors were stained with unlabeled primary and rhodamine-labeled secondary antibody (red fluorescence). Colocalization of receptors resulted in overlapping of red and green fluorescence, thus producing yellow color. Approximately 50 and 30% of CD4/CCR5 and CD4/CXCR4 double-positive cells, respectively, exhibited capping.

Table I. *Quantification of Receptor Capping*

			CD4/CCR5§		CD4/CXCR4‖		CD4/CXCR4¶	
	CCR5*	CXCR4‡	cont.	B-ol.	cont.	B-ol.	cont.	B-ol.
MIP-1β	61	15	–	–	–	–	40	9
$HIV\text{-}1_{92US660}$	–	–	53	10	–	–	–	–
$gp120_{JR\text{-}FL}$	–	–	66	–	14	–	39	3
SDF-1α	34	27	34	33	12	9	17	15
$HIV\text{-}1_{LAI}$	–	–	–	–	30	26	–	–
$gp120_{LAI}$	–	–	50	–	15	–	15	20

Cells were treated with the B-oligomer of PTX (1 nM) for 10 min (B-ol.) or left untreated (cont.), and then incubated with the indicated stimulant for 60 min. Capping was assayed by immunofluorescent microscopy (see Fig. 6). Approximately 200 cells were counted for each treatment. For some treatments, experiment was repeated two to three times with similar results.

*Percentage of $CCR5^+$ cells exhibiting capping. Approximately 30% of all cells were $CCR5^+$.

‡Percentage of $CXCR4^+$ cells exhibiting capping. Approximately 95% of all cells were $CXCR4^+$.

§ Percentage of $CD4^+/CCR5^+$ cells exhibiting capping. Approximately 10% of all cells were $CD4^+/CCR5^+$.

‖Percentage of $CD4^+/CXCR4^+$ cells exhibiting capping. Approximately 35% of all cells were $CD4^+/CXCR4^+$.

¶Percentage of $CCR5^+/CXCR4^+$ cells exhibiting capping. Approximately 30% of all cells were $CCR5^+/CXCR4^+$.

ing the notion that B-oligomer signals through its own receptor and this signaling is required for its anti-HIV activity.

Treatment of T lymphocytes with PTX or B-oligomer has been reported to rapidly increase PKC activity (30). To determine whether PKC was involved in the observed effects of B-oligomer on CCR5 function, we used two selective PKC inhibitors, Ro-31-8220 and Gö 6979. As shown in Fig. 5, Ro-31-8220 reversed the inhibitory effect of B-oligomer on entry of R5 HIV-1 (Fig. 5 A, panel a) and on Ca^{2+} flux induced by RANTES, another ligand for CCR5 (Fig. 5 B, panel a). On its own, Ro-31-8220 did not significantly alter either of those activities of CCR5 (Fig. 5, A and B, panel b). It also did not act upon entry of X4 HIV-1 (Fig. 5 A, panel b) or signaling by SDF-1α (Fig. 5 B, panels c and d), which are mediated by CXCR4 and are not affected by B-oligomer. A similar result was observed with a different PKC inhibitor, Gö 6979 (not shown). Taken together, these results indicate that PKC is involved in signal transduction from B-oligomer (and PTX) receptor to CCR5.

Our results thus far demonstrated that B-oligomer–treated cells did not signal after MIP-1β stimulation. We wished to demonstrate that signaling initiated by HIV-1 binding was also affected. Because our attempts to detect Ca^{2+} flux (14) or Pyk2 phosphorylation (13) in primary cells after inoculation with HIV-1 or gp120 were unsuccessful, we decided to use receptor capping as an indicator of HIV-1–induced signaling. Inoculation of PBMC cultures with either R5 (Fig. 6 A) or X4 (Fig. 6 B) HIV-1 strains, or their corresponding gp120s (Table I), induced a characteristic cocapping of chemokine receptors with CD4. The fact that we were able to detect capped CCR5 and CXCR4 using antibodies that compete with gp120 for chemokine receptor binding suggests that the observed signal came from free receptors that cocapped together with gp120-occupied receptors. Such interpretation is consistent with previously observed gp120-induced association of CD4 with several surface molecules, such as CD3, CD45RA, CD26, and HLA class I (41, 42), with RANTES-induced colocalization of CXCR4 and CD4 (43), and with cocapping of CCR2 and CCR5 in response to MCP-1 or RANTES (44).

Pretreatment of the cells with B-oligomer for 10 min prevented capping of HIV-1 receptors, but only when capping was induced by R5 HIV-1 (Fig. 6 A). This result indicates that capping is mediated by HIV-1–induced signaling from chemokine receptors, rather than from CD4, and that treatment with B-oligomer blocks this signal. To further illustrate the role of signaling from chemokine receptors in capping, we analyzed the effect of B-oligomer on capping induced by SDF-1α and MIP-1β. This analysis was confounded by rapid downregulation of CXCR4 or CCR5 after stimulation with SDF-1α or MIP-1β, respectively. Nevertheless, results presented in Table I demonstrate that B-oligomer blocked capping induced by MIP-1β, but not by SDF-1α. Similar to HIV-1– or gp120-induced polarization, the capping involved not only the ligand-specific receptor, but also other molecules, including CD4. This result suggests that signaling from chemokine receptors induces a major actin-dependent rearrangement of cellular membrane.

Taken together, the results presented in this report suggest that the inhibitory effect of PTX and its B-oligomer on HIV-1 infection of primary cells is mediated through desensitization of CCR5. Exposure to B-oligomer causes T cells to lose signaling activity associated with binding of the natural CCR5 ligand, MIP-1β, and to fail to cap after binding of R5 HIV-1; however, ligand-binding activity is

preserved. The role of receptor capping in HIV-1 infection of primary cells, and the nature of signals involved in the regulation of capping are now under investigation in our laboratory. It is clear that receptor capping is not required for binding of the virus, but it might be necessary for fusion with primary cells. In addition to the potential value of the B-oligomer as an anti-HIV agent, these studies may define new targets in the search for novel therapeutic approaches against HIV infection.

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