

Interference with the Signaling Capacity of CC Chemokine Receptor 5 Can Compromise Its Role as an HIV-1 Entry Coreceptor in Primary T Lymphocytes

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In this issue of *The Journal of Experimental Medicine* (1), Alfano et al. report the surprising results that pertussis toxin (PTX) as well as its cellular binding subunit, the B-oligomer, each blocked entry of monotropic (R5) strains of HIV-1 in primary T lymphocytes. Treatment of primary T cells with B-oligomer, unlike treatment of T cell lines, blocked calcium mobilization in response to the CCR5 chemokine ligand macrophage inflammatory protein-1 β (MIP-1 β), but had no effect on cell surface expression of CCR5 and binding of MIP-1 β or HIV-1 envelope gp120. Alfano et al. further demonstrate that B-oligomer blocks cocapping of CCR5 and CD4 induced by R5 HIV-1, but does not affect cocapping of CXCR4 and CD4 after incubation with T-tropic (X4) virus. They documented that the B-oligomer signals by itself and induces calcium mobilization in primary T lymphocytes. Based on these activating effects, the authors hypothesize that the inhibitory effect of B-oligomer is probably a consequence of cross-desensitization of CCR5, although the cellular receptor for B-oligomer has not yet been defined. These findings argue for the requirement of an intact signaling capacity for CCR5 to act as an HIV-1 fusion coreceptor in primary T cells and suggest a novel approach to the design of therapeutic inhibitors of CCR5. There are a number of research reports that provide added insights concerning the mechanistic basis for these provocative findings.

The current model of the HIV-1 fusion predicts that after binding of the envelope gp120 to the CD4 molecule, a conformational change in gp120 ensues, which enables its critical domain (or domains) to engage one of the chemokine receptors. This process is necessary for the fusogenic conversion of HIV-1 gp41 and the exposure of its fusion peptide domain (2, 3). The usage of chemokine receptors by HIV-1 Env and the ability of receptor-specific chemokine ligands to prevent HIV-1 fusion and infection raised the question whether the process of HIV-1 fusion is passive or requires chemokine receptor signals. A number of studies of CCR5 have suggested that chemokine receptor signaling may be dispensable for its role as an HIV-1 fusion coreceptor (4–6). For example, PTX did not interfere with the capacity of HIV-1 to infect T cell lines *in vitro*. Furthermore, truncation of the COOH tail of CCR5 or mutation of the highly conserved aspartate-arginine-tyrosine (DRY) sequence in the second cytoplasmic loop each ef-

fectively blocked chemokine-dependent activation of classical second messengers, intracellular calcium mobilization, and cell migration in response to CCR5 chemokine ligands. Yet none of these mutations altered the ability of CCR5 to support HIV-1 entry (7). Similar truncations or mutations of CXCR4 also did not alter its function as a coreceptor for the X4 HIV-1 (8). These results lead to the conclusion that G protein-coupled signaling, a consequence of CCR5 and CXCR4 activation by their chemokine ligands, was not involved in the process of HIV-1 fusion. Thus, CCR5 or CXCR4 was considered to merely play a passive role as an anchor for HIV-1 envelope protein based on their association with the CD4 molecules on the cell membrane (9, 10).

Although HIV-1 apparently does not require an intact chemokine coreceptor signaling capacity for cell entry, several lines of evidence have shown that HIV-1 envelope (Env) proteins nevertheless activate G proteins through their interaction with CCR5 or CXCR4. The chemotactic and calcium mobilizing activity of soluble gp120 for CD4⁺ T lymphocytes and monocytes were documented more than a decade ago (11). Recently, Weissman et al. reported that mammalian cell-derived recombinant R5 HIV-1 Env was able to induce Ca²⁺ flux and migration in cultured primary CD4⁺ T cells (12). Since the signaling of HIV-1 Env in CD4⁺ T cells could be specifically desensitized by the CCR5 ligand MIP-1 β , it was concluded that this R5 HIV-1 Env activated CCR5 in the presence of CD4 (12). Unlike R5 HIV-1 Env, the X4 HIV-1 gp120 seems to be able to activate CXCR4 in the absence of CD4 by inducing migration and Ca²⁺ flux in both CD4⁺ and CD8⁺ T lymphocytes (13, 14).

There is considerable evidence that HIV-1 and envelope proteins have the capacity to induce signal transduction events independent of G_i proteins. HIV-1 gp120 of both R5 and X4 strains has been reported to induce phosphorylation of tyrosine kinase Pyk2 in myeloid cell lines (15). HIV-1 Env also inhibits the expression and function of a number of chemoattractant receptors, including those for chemokines and the bacterial chemotactic formyl peptide fMLP on monocytes, through a CD4- and protein kinase C-mediated receptor “desensitization” (16). More recently, an X4 HIV-1 gp120 was also found to induce CXCR4 phosphorylation and to downregulate CXCR4 in

human CD4⁺ T cells through activation of an src-like tyrosine kinase p56^{lck} (17). Consequently, the interaction of HIV-1 Env with human cells can elicit a variety of signaling events that involve both tyrosine kinase- and G protein-associated signals.

Although it is well known that HIV-1 Env induces signal transduction in host cells, such signaling events are not thought to play a role in HIV-1 fusion/entry, but to contribute to postfusion effects of HIV-1 (12–14). However, evidence is accumulating that chemokine receptor signaling triggered by direct or indirect interaction with HIV-1 Env may have a profound impact on the fusion/entry of HIV-1. Guntermann et al. observed that preincubation of IL-2-treated PBMCs with PTX markedly inhibited infection of the cells by X4 HIV-1, while the cells maintained a normal level of expression of CD4 and CXCR4 on the surface (18). The authors suggest that G_i protein-mediated receptor signaling may be necessary for the competence of CXCR4 as a viral fusion coreceptor in primary T cells. Although attribution of the anti-HIV-1 effect of PTX holotoxin to its inhibition of G_i protein-mediated signaling is inappropriate, Guntermann et al. provided the first evidence that perturbation of the signaling capacity of a coreceptor in primary T cells could effectively block HIV-1 infection. This is in contrast with a previous report in which T lymphocyte cell line PM1 treated with PTX was still sensitive to HIV-1 infection, albeit with a reduced production of p24 antigen levels 10 d after infection (19). Alfano et al. point out that the failure of PTX to block HIV-1 infection of PM1 cells is presumably based on the inability of these cells to generate signals in response to the PTX B-oligomer (1). Additional evidence for a signal-dependent entry of HIV-1 was shown by the inhibition of viral entry in PHA-stimulated PBMCs by cytochalasin D, a specific inhibitor of cytoskeletal F-actin polymerization that does not affect cell surface expression of CD4 and HIV-1 coreceptors (20). Alfano et al. confirm that PTX inhibits infection of activated PBMCs by R5 HIV-1 and indicated that the inhibition of HIV-1 infection by PTX occurred at the stage of viral entry (1). Furthermore, Alfano et al. demonstrate that the anti-HIV-1 activity of PTX was in fact mediated by a subunit of PTX, namely the B-oligomer, which is responsible for the binding of PTX holotoxin to the target cells and exhibits a number of biological activities on eukaryotic cells independent of the G_i protein inhibitory effect of PTX holotoxin (21). On the other hand, the entry of X4 HIV-1 into PBMCs was affected by neither PTX holotoxin nor B-oligomer, suggesting that the inhibitory effect is CCR5 specific.

PTX is the major virulence factor produced by the Gram-negative bacterium *Bordetella pertussis*, the etiological agent of whooping cough. PTX is a heterohexameric protein and is functionally divided into A (protomer) and B (oligomer) domains (21). The A-protomer consists of an S1 subunit, and the B-oligomer is composed of two dimers (S2-S4 and S3-S4) joined together by an S5 subunit (Fig. 1). The subunits are connected by noncovalent interactions. The A-protomer of PTX has ADP ribosyltransferase activ-

ity, whereas the B-oligomer confers cell surface binding property on PTX. Due to the ADP-ribosylation activity of the A-protomer, PTX has been a very useful pharmacological tool for the identification of inhibitory guanine nucleotide binding (G_i) proteins in the plasma membrane that are coupled to seven-transmembrane (STM) chemokine receptors, including CCR5 and CXCR4. However, A-protomer per se does not bind to the cells and therefore is inactive in the absence of B-oligomer. In addition to the ADP-ribosylation of G_i-like proteins, PTX has been demonstrated to elicit a number of biological events in T lymphocytes (22, 23), adipocytes (24), platelets (25), and macrophages (26). A recent study indicated that PTX potentiates Th1 and Th2 responses to coinjected antigen by enhancing cytokine production and expression of costimulatory molecules (27). Since these effects of PTX were displayed largely either by biochemically purified B-oligomer or by PTX deprived of enzymatic activity yet retaining cell binding and signaling capacity based on phospholipase C and tyrosine phosphorylation pathways, it is postulated that cellular binding sites or receptor(s) for B-oligomer are critical for the interaction of PTX with its target cells. Efforts to characterize PTX binding sites have yielded several surface glycoproteins in different cell types that may generate early signaling events (21). However, whether PTX binds to different sites, resulting in either early cell signaling or G protein inactivation, or binds to the same site, resulting in both these stimulatory and inhibitory effects, remains to be elucidated. Another concern is whether PTX B-oligomer-mediated cellular transmembrane signal transduction affects the subsequent ADP-ribosylation of G_i-like proteins. Thus, a simple demonstration of the inhibition by PTX of a cellular response to an agonist may not provide a final proof that the agonist effect involves G_i protein. This argument appears reasonable, as demonstrated by Alfano et al. (1), who show that the inhibition of R5 HIV-1 fusion via CCR5 by PTX can be fully accounted for by the potent cell-activating effects of B-oligomer and the loss of the capacity of CCR5 to act as a viral fusion coreceptor.

The agonist-induced phosphorylation of STM receptors such as CCR5 and CXCR4 can result in homologous desensitization and internalization of the receptors (28, 29). Homologous desensitization occurs in receptors in an agonist-occupied state and involves phosphorylation by G protein-coupled receptor kinases. These phosphorylated receptors associate with members of the arrestin family, resulting in a decreased affinity of the receptor for G proteins and increased receptor internalization (30). Heterologous desensitization is characterized by the loss of receptor function after phosphorylation induced by second messenger-activated kinases such as protein kinase (PK)C or PKA, as a result of activation of different receptors or signaling processes (30). Heterologous desensitization does not involve agonist occupancy of the receptor and may not lead to arrestin-mediated receptor internalization. Since heterologous desensitization has been reported to occur between a number of STM receptors, it has been postulated to play an important role in an orchestrated host cell response in

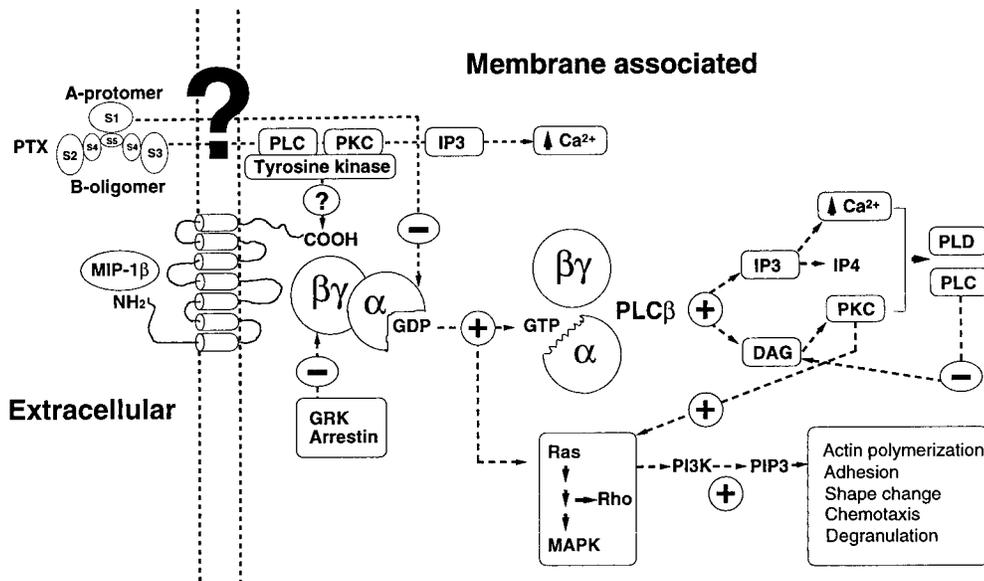


Figure 1. Putative effect of PTX on cell activation. PTX binds to a yet to be defined cell surface receptor through the sub-unit B-oligomer, which enables the entry of the A-protomer. The A-protomer inhibits G_i protein activation of CCR5 in response to chemokines, whereas the B-oligomer elicits a series of cellular signaling events that may lead to desensitization of CCR5. DAG, diacylglycerol; GD(T)P, guanosine di(tri)phosphate; GRK, G protein-coupled receptor kinase; IP, inositol phosphate; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol trisphosphate; PLC(D), phospholipase C(D).

the presence of multiple stimulants. For example, activation of two STM opiate receptors, δ and μ , resulted in a decrease in phagocyte migration to a number of chemokines (31). Furthermore, activation of δ and μ opiate receptors induced chemokine receptor phosphorylation without a change in the level of cell surface expression or agonist-induced calcium mobilization (31). In the study by Alfano et al. (1), B-oligomer does not directly compete with chemokine MIP-1 β for binding to CCR5-expressing PBMCs, suggesting that B-oligomer does not use CCR5 as its functional receptor. However, after stimulation with B-oligomer the cells showed significantly reduced calcium mobilization in response to MIP-1 β . Although the cells maintained a normal level of expression of CCR5 on the cell surface after treatment with B-oligomer, C5 HIV-1 Env gp120 could no longer induce cocapping of CD4 and CCR5, a critical step for viral entry. These results indicate that activation of CCR5 by B-oligomer impairs normal signaling of CCR5 followed by a loss of its function as a fusion coreceptor for HIV-1. Since B-oligomer has been reported to activate PKC in human leukocytes through putative cell surface receptors, it is plausible that the attenuation of CCR5 functioning by B-oligomer may be a consequence of heterologous receptor desensitization (Fig. 1). Despite the fact that the B-oligomer only inhibits the entry of R5, but not X4, HIV-1 virus (1), productive infection of X4 HIV-1 was also inhibited by long-term exposure of cells to nontoxic concentrations of B-oligomer, as shown by Alfano et al. Whether this is due to the disturbance of CXCR4-related late stage cell signaling by an as yet unknown mechanism remains to be determined.

The discovery of chemokine receptors as HIV-1 fusion coreceptors has prompted intensified efforts to search for novel anti-HIV-1 therapeutics by developing chemokine-

receptor antagonists. In addition to the specific chemokine ligands for the coreceptors, modified chemokines such as Met- or AOP-RANTES are more potent competitors of CCR5 and more effectively inhibit HIV-1 fusion (32, 33). Other synthetic oligopeptide and compounds have also been reported to be highly efficacious in disrupting CXCR4-mediated HIV-1 fusion (34, 35). An analogue of the antibiotic Distamycin, NSC651016, by binding to and downregulating CXCR4 and CCR5, was shown to inhibit cell migration induced by CXCR4 or CCR5 chemokine ligands and infection by both R5 and X4 HIV-1 (36). These antagonists specifically interact with the fusion coreceptors and competitively inhibit the binding of HIV-1 Env to the cell surface. An alternative approach to inhibiting HIV-1 infection is to reduce cell surface expression of HIV-1 coreceptors. This approach is validated by the fact that subjects with an allotypic variant of CCR5 that results in failure to express this coreceptor on cell surface are largely resistant to HIV-1 infection (37, 38). The anti-HIV-1 activity of chemokines and their analogues also is in part attributable to a rapid internalization of the fusin coreceptors. In addition, chemokines RANTES, MIP-1 α , or SDF-1 α that are genetically engineered to express an endoplasmic reticulum retrieval peptide, KDEL, at the COOH termini can retain newly synthesized coreceptors CCR5 or CXCR4 intracellularly, possibly by forming complexes (39, 40). Lymphocytes transfected with these modified chemokines (termed intrakines) were viable and resistant to HIV-1 infection. Although many questions remain to be resolved, the effects of the PTX B-oligomer observed by Alfano et al. suggest an additional approach to developing anti-HIV-1 therapeutics with the capacity to heterologously desensitize coreceptor signaling.

The authors thank Dr. F. Ruscetti for critical review of this manuscript.

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Submitted: 14 June 1999 Accepted: 28 July 1999

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