β-Chemokine MDC and HIV-1 Infection

The human immunodeficiency virus type-1 (HIV-1) uses the chemokine receptors CCR5 or CXCR4 (in conjunction with CD4) to enter healthy cells, and the chemokine ligands to these receptors generally block virus infection (1–3). R. Pal et al. studied a truncated form of the β-chemokine macrophage-derived chemokine (MDC), which lacked the first two NH2-terminal amino acids [(−2)MDC] that they purified from an immortalized CD8+ T-cell clone. They found that (−2)MDC inhibited both R5 and X4 HIV-1 strains in vitro (4). The effects of full-length MDC, a CCR4 ligand (5), on HIV-1 infection have not been described.

To investigate the mechanism by which a chemokine to CCR4 [which is not an HIV-1 coreceptor (6, 7)] could inhibit infection by diverse HIV-1 isolates, we prepared MDC with the use of a bacterial expression system that we have used successfully to produce other anti-viral chemokines. MDC produced in this way was a potent CCR4-ligand, which induced a calcium flux in cells that transiently express CCR4 (Fig. 1, A to C), naturally CCR4-positive cells such as Jurkat (Fig. 1B) and UT-7 (8), and activated peripheral blood mononuclear cells (PBMCs) (Fig. 1, C and D). MDC did not stimulate a calcium flux when added to cells that transiently express other chemokine or orphan receptors, including CCR1, -2, -3, -5, or -8, CXCR2, CXCR4, GPR1, GPR15, and STRL33 (8). Further, addition of MDC to PBMCs desensitized their subsequent response to the CCR4 ligand TARC (Fig. 1D). MDC did not desensitize subsequent CCR5 or CXCR4 responses, which suggests that it does not down-regulate these receptors (Fig. 1, B and C). MDC was also a potent chemotactrant, as previously described (9). By contrast, recombinant (−2)MDC did not induce a Ca2+ flux in multiple cell types that express CCR4 or other receptors, including PBMCs (Fig. 1, A to C), and it was 1000-fold less effective than full-length MDC in chemotaxis assays. We also found that synthetic MDC, while biologically active, lacked anti-viral properties. We conclude that recombinant (−2)MDC, produced by the same method used to generate biologically active MDC (and also other chemokines such as RANTES and SDF-1), is neither a CCR4 ligand nor antagonist, nor an agonist for the relatively large number of chemokine and orphan receptors tested or for receptors present on PBMCs.

Even high concentrations of MDC did not inhibit productive infection of PBMCs (Fig. 2A), or macrophages (8) by X4, R5, or R5X4 HIV-1 strains. MDC also did not inhibit virus entry into PBMCs as determined by a PCR entry assay; also, it did not inhibit infection of multiple cell lines by pseudotyped reporter viruses bearing X4, R5, or R5X4 Env proteins, including cell lines that express CCR4 in addition to coreceptors such as CXCR4 and CCR5 (8). MDC did not inhibit fusion of cells expressing either X4 or R5 Env proteins with PHA- or PHA+IL-2–activated PBMCs (8), PHA-activated primary CD4+ T-cells (Fig. 2B), macrophages (Fig. 2B), PM-1, or UT7 cells (8) with the use of a well-characterized gene reporter fusion assay (10). By contrast, SDF-1 inhibited fusion by X4 Env proteins and RANTES inhibited fusion by R5 Env proteins. Recombinant (−2)MDC also did not inhibit infection of PBMCs by X4, R5, or R5X4 virus strains, whereas AOP-RANTES inhibited CCR5–dependent virus infection in the same cells (Fig. 2A).

MDC has not been shown to interact with any known HIV coreceptor, which suggests that any anti-viral activity it may have is likely to be indirect, perhaps as a result of intracellular signaling. The recombinant MDC we produced, however, did not inhibit any virus strain tested, despite being fully capable of signaling through CCR4, nor did it affect CXCR4 or CCR5. The recombinant (−2)MDC was also inactive against HIV-1 and did not induce signals in PBMCs or by any receptor we tested it against in cell lines. The reasons underlying the discrepancies between our results and those of Pal et al. (4) are not clear. In our study, we used recombinant chemokines, whereas Pal et al. purified (−2)MDC from CDB7 cell–conditioned medium. Each of these approaches has potential weaknesses. In principle, our recombinant (−2)MDC might fold or be processed differently from (−2)MDC made in CDB7 cells, preventing it from having activity against HIV-1. Recombinant RANTES and SDF-1 made by the same method, however, retain antiviral activity, and our recombinant MDC was a highly effective CCR4 agonist. Conversely, (−2)MDC isolated from conditioned medium may not have the purity of a recombinant protein, raising the possibility that the antiviral activity noted by Pal et al. in their (−2)MDC preparation might be attributable to an undetected contaminant. Further studies might resolve these issues.

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Fig. 2. Virus infection and cell-cell fusion assays. (A) PBMC were isolated by Ficoll hypaque and stimulated with 250 ng/ml PHA for 72 hours, and subsequently expanded in the presence of 50 U/ml IL-2 for 48 hours before infection by HIV-1 IIIB [multiplicity of infection (m.o.i.) 0.2], HIV-1 Ba-L (m.o.i. 0.2), or HIV-1 89.6 (m.o.i. 0.2) in the presence of 50 U/ml IL-2 alone (that is, "None") or in combination with 500 ng/ml MDC (donor shown on the left), 500 ng/ml (-2)MDC (donor shown on the right) or 100 ng/ml AOP-RANTES (both donors). Cultures were sampled every 3 days, at which time medium with cytokines, or chemokines, or both was replenished. Reverse transcriptase (RT) activity within supernatants was determined against a standard curve of recombinant HIV-1 BH10RT (NIH AIDS Reference Reagent Program). Data is shown for day 10 after infection as picograms of RT activity per 0.01 ml. Similar results were obtained with either MDC or (-2)MDC with the use of the same infection protocol with cells from different donors (B). To measure cell-cell fusion, HeLa cells were infected with recombinant vaccinia viruses expressing the desired HIV-1 Env protein, as well as with vCB21R, a recombinant virus containing the E. coli lacZ gene under the control of the T7 promoter. HeLa cells were mixed with either macrophages or CD4+ T cells. Both cell types were infected with a recombinant vaccinia virus expressing T7 RNA polymerase. Macrophages were prepared from elutriated monocytes by 14 days of differentiation. Purified types were infected with a recombinant vaccinia virus expressing T7 RNA polymerase.

Supernatants of activated CD8+ T lymphocytes suppress HIV without cell lysis. The CC chemokines RANTES, MIP-1α, and MIP-1β, are known to prevent the entry into healthy cells and consequent replication of CCR5-dependent (R5) HIV strains. Other factors, however, are thought to be involved, because residual suppressive activity remains after blockade of these chemokines with selective antibodies. Moreover, the presence of these chemokines does not explain the capacity of the CD8+ T cell supernatants, which do not contain SDF-1 (2), to inhibit entry and replication of CXCR4-dependent (X4) HIV strains.

R. Pal et al. report (3) a CD8+ T lymphocyte–derived factor that appeared to suppress X4 or R5 HIV isolates; they found that this factor corresponded to a truncated form of MDC, MDC(3-69), which lacked two NH2-terminal residues (3). MDC, a CC chemokine with the NH2-terminal sequence GPIGANGMEDSVCC... was recently isolated from human macrophages (4). We have synthesized wild-type MDC and MDC(3-69) and have tested their activities in comparison with that of TARC, which, like MDC, is selective of the CC chemokine receptor 4, CCR4 (5). MDC- and TARC-induced chemotaxis and Ca2+ mobilization in cells bearing CCR4 and showed cross-desensitization (Fig. 1). In contrast, MDC(3-69) was inactive and did not desensitize or block CCR4. Furthermore, MDC did not cause down-regulation of CCR5 or CXCR4, in contrast to their respective ligands, MIP-1β and SDF-1. HIV suppression in activated, CD8+ T lymphocyte–depleted PBMCs was also tested. MDC and MDC(3-69), as well as TARC, had no activity, whereas marked suppression of either X4 or R5 strains was obtained with SDF-1 and MIP-1β, respectively. As with single tropic X4 or R5 viruses, MDC(3-69) also did not prevent replication of dual tropic R5X4 HIV isolates. Our results show that MDC, MDC(3-69), and TARC lack suppressive activity against CCR5 or CXCR4-dependent HIV strains, or both.

We find no evidence for an interaction of MDC or MDC(3-69) with either CXCR4 or CCR5, and for the HIV isolates tested we confirm previous observations showing that CCR4, the selective receptor for MDC and TARC, does not function as an HIV coreceptor (6). These results do not support the notion that MDC or MDC(3-69) is responsible for HIV inhibitory activity of CD8+ T lymphocyte supernatants.

References and Notes
8. B. Lee et al., unpublished results.
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attached to the surface of magnetic beads) were infected with 100 to 200 TCID50 of X4 (NL4-3), R5 (BaL) antigen. Results corresponding to day 10 after infection are shown. All the isolates were tested at least incorporated 200 nM of chemokines. Viral replication was assessed by measuring soluble HIV p24 gag throughout the culture period. Culture supernatants were collected every 3 days, and medium changes were recorded. The same results were obtained when MDC(3-69) was used at 1,000 nM. (C) Chemotaxis of CEM cells induced by MDC (●), MDC(3-69) (○), and TARC (▲). For methods used in A to C, see (7). (D) Down-regulation of CXCR4 and CCR5 in phytohemagglutinin (PHA)-and IL2-induced blood CD4+ T cells. Surface expression (relative units) of the receptors is shown on in-duction with SDF-1, MIP-1β, MDC, or MDC(3-69), as previously described (8). CXCR4 and CCR5 were detected with the use of the monoclonal antibodies 12G5 and 7E10, respectively (NIH AIDS Re-pository Reagent Program). (E) Ef-fect of 200 nM SDF-1, MIP-1β, MDC, MDC(3-69), or TARC on HIV replication. 2×106 PHA- and IL2-activated PBMCs depleted of CD8+ T lymphocytes (antibody to CD8 attached to the surface of magnetic beads) were infected with 100 to 200 TCID50 of X4 (NL4-3), R5 (BaL and SF162), or RSX4 (SF2) HIV isolates. Chemokines were added before infection and maintained throughout the culture period. Culture supernatants were collected every 3 days, and medium changes incorporated 200 nM of chemokines. Viral replication was assessed by measuring soluble HIV p24 gag antigen. Results corresponding to day 10 after infection are shown. All the isolates were tested at least twice with the use of different healthy blood donors. Representative results are shown.

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Table 1. Isoforms of native MDC.

<table>
<thead>
<tr>
<th>Molecular masses by mass spectrometry (Da)</th>
<th>Predicted sequence</th>
<th>Truncation</th>
<th>NH2-terminal sequences obtained</th>
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<tbody>
<tr>
<td>7956†</td>
<td>YGANMEDSVCCRD.</td>
<td>(−2)</td>
<td>yes</td>
</tr>
<tr>
<td>7735†</td>
<td>ANMEDSVCCRD.</td>
<td>(−4)</td>
<td>yes</td>
</tr>
<tr>
<td>7277†</td>
<td>DSVCCRD.</td>
<td>(−8)</td>
<td>no</td>
</tr>
</tbody>
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MALDI-TOF mass spectrometry was performed on a PerSeptive Biosystems Voyager mass spectrometer (PerSeptive Biosystems, Framingham, MA) with the use of 3.5-dimethoxy-4-hydroxycinnamic acid (Sigma, St. Louis, MO) as a matrix.

*An isoform with the sequence PYGANMED... was also detected in our original sequence analyses.

Tentatively identified as MDC plus Na+.

References
2. S. F. Lacey, C. B. McDanal, R. Horuk, M. L. Greenberg,

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MDC isoforms consistently stimulates cytoplasmic calcium mobilization in activated PBMCs after long- or short-term culture (1). The activity is equivalent to that which is seen with the same concentration of synthetic full-length MDC (Fig. 1A), which strongly indicates that the native MDC isoforms, or one of them, is functional. The alternative explanation, which seems less likely, is the existence of a contaminant, undetected by sequencing or mass spectrometry, that triggers G-protein–coupled receptors to the same level as synthetic MDC. However, a monoclonal antibody against MDC that blocks signaling inhibited the induction of calcium mobilization by native MDC (Fig. 1B), although it did not directly block the antiviral effect (2). These results indicate that the signaling activity in our preparations is not a result of a contaminant that induces calcium mobilization, but is inconclusive with respect to antiviral activity. One explanation for the differential inhibition is that the monoclonal antibody does not recognize the antiviral domain or isoform. Further experiments with additional monoclonal and polyclonal antibodies might resolve this issue.

The major question now centers on which molecule in the native MDC preparations is responsible for the antiviral effect. On the basis of available evidence, it seems reasonable to eliminate full-length MDC from consideration as the major antiviral species because it is functional with respect to receptor activation. It is unclear, however, whether the −2 isoform can be characterized as inactive. Given our results with native material, at this time we cannot conclude that the −2 isoform is naturally inert. It seems equally possible that the −2 MDC isoform is at least partially functional, but prone to misfold and lose activity under certain conditions. Thus, it may not be unexpected that such an inert form of −2 MDC lacks antiviral activity. We also cannot conclude that the −2 isoform does not bind known HIV coreceptors because one commercial preparation (Peprotech, Rocky Hill, New Jersey) competes with MIP-1α for binding to CCR5, albeit at high concentrations (IC_{50} for MDC 202 nM). These results (11) are not necessarily at odds with those of Arenzana-Seisdafer et al. and Lee et al., because it is possible that the isoform exhibits promiscuous but nonfunctional receptor interactions that may not have been detectable in their assay systems. Such interactions were demonstrated for the human herpesvirus 8 chemokine analog, vMIP-II (10, 12). Promiscuous receptor binding is consistent with the broad antiviral effect we observed; however, material we obtained from this source before commercialization was not antiviral in our assays (2). We are currently evaluating more recent preparations of this material in order to address this issue.

It is also possible that the activities we observe with the native MDC are a result of other isoforms we have not yet analyzed separately. Finally, although there is no conclusive evidence, it is possible that MDC may be closely associated with a cryptic factor that is the determinant for antiviral activity.

In summary, we agree that no recombinant form of MDC has yet shown antiviral activity, while the native truncated forms show both antiviral activity and the capacity to stimulate cytoplasmic calcium mobilization. Thus, the −4 and −8 forms, and even the −2 form, cannot be definitively excluded as the active molecule or molecules. Further experimentation is required to resolve these issues.

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Fig. 1. Intracellular Ca^{2+} mobilization induced by native and synthetic MDC. (A) Primary PBMC were activated for 72 hours in 5 μg/ml phytohemagglutinin (PHA) and 20 U/ml recombinant human IL-2 (rIL-2; Boehringer-Mannheim, Indianapolis, Indiana) and then cultured in rIL-2. Cells were treated with either synthetic or native MDC (33 nM each) and assayed as described previously (1). Data were acquired by a FACS Calibur (Becton-Dickinson, San Jose, California) flow cytometer, gating cells by forward and side scatter properties. Ca^{2+} mobilization was determined by analysis in a two-parameter density plot of linear emission at 530 nm collected in the FL-1 window over time. Time units are equal to 0.1 seconds. (B) Assay was repeated with the use of synthetic or native MDC (3 nM each) that was untreated or treated with a five-fold molar excess of monoclonal antibody against MDC for 1 hour at 4°C. Time units shown are equal to 0.05 seconds.
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References and Notes

2. A. Devico et al., unpublished results.
4. A 1:30 dilution of the preparation (1.1 μg/ml) suppressed HIV-1 infection by 81 percent in assays performed with CD8+ T cell–depleted PBMCs as previously described (1).
11. P. Gray and V. Schweickart, personal communication.
13. We thank P. Gray and D. Chantry (Icos Corporation, Bothell, WA) for kindly providing synthetic MDC and antibodies against MDC and N. Ambulos (Department of Microbiology and Immunology, University of Maryland, Baltimore, MD) and D. Barofsky (Department of Chemistry, Oregon State University, Corvalis, OR) for help in performing mass spectrometry and analyzing the data.

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