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ORIGINAL INVESTIGATION

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Role of curdlan sulfate in the production of β-chemokines and interleukin-16

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Abstract The blocking effect of curdlan sulfate (CRDS) on human immunodeficiency virus (HIV) infection has been thought to be related to inhibition of the binding of HIV-1 envelope glycoprotein (gp120) and CD4 molecules. However, recent reports have indicated that blocking the binding of gp120 to CD4 by CRDS only makes a small contribution to the inhibition of HIV-1 infection. We report here that the effect of CRDS on the production of β-chemokines and cytokines might be important in the inhibition of HIV-1 infection, in addition to interference with the binding of gp120 to $CD4^+$ cells.

Key words HIV-1 · gp120 · Curdlan sulfate · Chemokine · Interleukin-16

Introduction

The envelope glycoprotein of HIV-1 (gp120) is known to bind to CD4 molecules on T cells and macrophages [8, 26]. Therefore, blocking of viral attachment via gp120 to CD4 cells has been suggested as one therapeutic strategy for HIV-1 infection [6, 29, 35]. Sulfated polysaccharides have been reported to inhibit HIV-1 infection by blocking this attachment. In particular, curdlan sulfate (CRDS), a sulfated polysaccharide with 1,3-β-D-glucan as the main chain, has been shown to potently inhibit HIV-1 infection in vitro [1, 2, 16, 21, 38]. However, recent reports have indicated that the marked in vitro inhibitory effect of CRDS on HIV-1 infection is not fully explained by the blocking of attachment to CD4 molecules and that some other action of CRDS may also be involved [16, 38].

It has been reported that β-chemokine receptors are coreceptors for HIV and some β-chemokines can inhibit HIV infection in vitro [7, 9–11, 15, 32]. In addition, interleukin (IL)-16 has been reported to inhibit the expression of messenger RNA of HIV-1 in vitro [3, 40]. Although the inhibitory effect of IL-16 on HIV-1 infection is still controversial, this may be dependent on differences in the systems used [3, 13, 40]. We have recently shown that CRDS can inhibit not only virus attachment but also gp120-mediated tumor necrosis factor (TNF)-α production [38]. The latter action of CRDS may be involved in its inhibition of HIV-1 infection because TNF-α can promote HIV-1 replication [33]. In this study, we investigated the effect of CRDS on the production of β-chemokines and IL-16 in relation to its blocking of HIV infection.

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Materials and methods

Cells and reagents

Peripheral blood mononuclear cells (PBMC) were separated from normal human blood by centrifugation on a Ficoll-Paque cushion. Cultures were performed in a 5% CO_2 incubator at 37 °C using RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Gibco Laboratories, Grand Island, N.Y.).

CRDS (79±0.6 kDa with a sulfur content of 15.2±0.3%) was provided by Ajinomoto Co. (Tokyo, Japan).

Unlabeled and fluorescein isothiocyanate (FITC)-labeled recombinant HIV-1 envelope glycoprotein gp120 ($HIV\text{-}1_{IIIB}$) were obtained from a baculovirus expression system, and showed >90% purity as estimated by analysis of Comassie blue-stained sodium dodecyl sulfate-polyacrylamide gels (Intracel Co., Issaquah, Wash.). Our previ-

ous experiments showed that 1 μg/ml of the glycoprotein was sufficient to occupy all the cell surface receptors [19, 20, 31, 36–38], so this concentration was used in the present study.

Flow cytometry

To observe the blocking effect of CRDS on the binding of gp120, PBMC (1×10^6 cells/ml per well) were cultured with CRDS for 24 h and incubated with FITC-labeled gp120 (1 μg/ml) for 2 h. The cell analysis was performed using a FACStar plus (Becton Dickinson, Mountain View, Calif.).

Chemokine and cytokine production

To investigate the effect of CRDS on the production of chemokines and cytokines, PBMC (2×10^5 cells/ml per well) were treated with CRDS for 24 h and then incubated for 24 h with or without FITC-unlabeled-gp120 (1 μg/ml). In some experiments, lipopolysaccharide (LPS; Sigma, St. Louis, Mo.) (1 μg/ml) was used as a stimulator instead of gp120. The levels of macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemoattractant protein (MCP)-1, RANTES (regulated upon activation, normal T cell expressed and secreted), and IL-16 in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA; R&D system, Minneapolis, Minn. and Biosours International, Camarillo, Calif.) [31].

Statistical analysis

Statistical analysis was performed using Student's *t*-test.

Results

Effect of CRDS on the binding of HIV-1 gp120 to cells

Figure 1 shows the inhibitory effect of CRDS on the binding of gp120 to PBMC. This inhibition was concentration dependent. CRDS did not have any influence on the expression of CD4 molecules on cells, as we previously reported [38].

Effect of CRDS on the production of β-chemokines and IL-16

Production of β-chemokines is known to be induced by gp120 stimulation [31]. Gp120-mediated MIP-1α, MIP-1β, and MCP-1 productions was inhibited by CRDS (Fig. 2 a–c). In contrast, production of RANTES by gp120-stimulated PBMC increased in the presence of CRDS (Fig. 2 d). These effects of CRDS on β-chemokine production were concentration dependent (Fig. 2). CRDS exerted no influence on cell growth and viability at the concentrations used in this experiment (data not shown). We next examined whether there was an inhibitory effect of CRDS on the production of MIP-1α, MIP-1β, and MCP-1 when production was induced by LPS stimulation instead of gp120. As shown in Fig. 3, CRDS inhibited the production of MIP-1α, MIP-1β, and MCP-1 by PBMC stimulated with LPS. In addition, CRDS directly enhanced the production of RANTES (but not MIP-1α, MIP-1β, and MCP-1), even in the absence of gp120, in a concentration-dependent fashion (Fig. 4). As shown in Fig. 5, CRDS increased the production of IL-16 by PBMC both in the presence and absence of gp120, although gp120 alone did not increase IL-16 production.

Discussion

Recent treatments using nucleoside analogues, protease inhibitors, or combinations of these agents have proved extremely useful for suppressing virus load expansion. However, HIV-1 can develop resistance to these agents [14], so the strategy of blocking viral attachment is still potentially important as an anti-retroviral therapy that prevents infection. With respect to interference with viral attachment to CD4, soluble recombinant CD4, as well as sulfated polysaccharides such as dextran sulfate (1-6-α-D-glucan), have been tested in clinical trials on HIV-1-infected patients [12, 18]. Despite an inhibitory effect on HIV-1 infection in vitro [6, 29, 35], these agents are not widely employed because of their insufficient anti-HIV-1 activity in vivo and various adverse effects including thrombocytopenia [12,

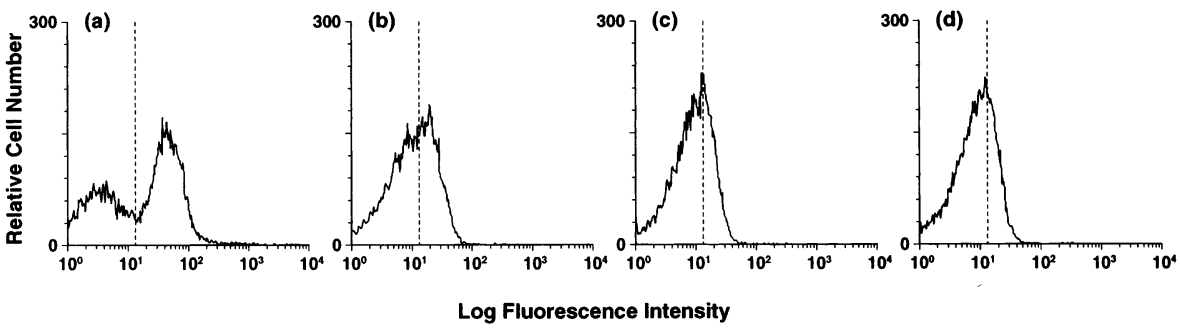

Fig. 1 a–d Blocking effect of CRDS on the binding of gp120. Concentrations of CRDS are 0 μg/ml (**a**), 1 μg/ml (**b**), 10 μg/ml (**c**), and 100 μg/ml (**d**). The *broken lines* indicate the autofluorescence threshold in each histogram. Percent inhibition of gp120 binding to cells by the indicated concentrations of CRDS, which was obtained by comparsion of the mean fluorescence intensity of gp120 binding without and with CRDS treatment, were as follows: **a** 0% (control); **b** 21%; **c** 36.5%, **d** 45.5%. *CRDS* curdlan sulfate

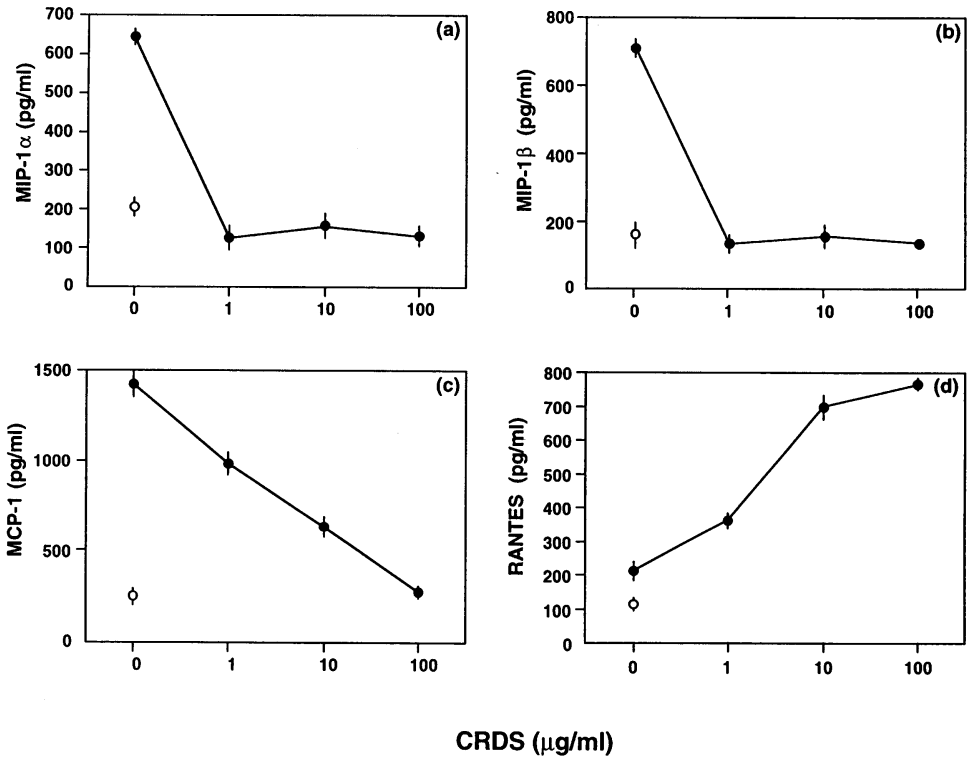

Fig. 2 Effect of CRDS on the production of β-chemokines (**a** MIP-1α; **b** MIP-1β; **c** MCP-1; **d** RANTES) by cells cultured with (●) and without (○) gp120. There were significant differences of β-chemokine levels between cultures without CRDS and with CRDS (100 μg/ml) in **a–d** (P<0.01)

18]. CRDS is known to strongly inhibit HIV-1 infection in vitro, and this agent is expected to be more effective and less toxic than dextran sulfate [1, 2, 16, 21, 38]. Previous studies using reverse transcriptase (RT) activity and a p24 antigen assay [1, 2, 16, 21] have shown approximately 100% inhibition of HIV-1 infection of PBMC by CRDS (100 μg/ml) in vitro. However, it is difficult to explain this marked in vitro inhibitory effect of CRDS on HIV-1 infection solely on the basis of the blocking of gp120-CD4 attachment, because the percent inhibition of gp120 binding by CRDS (100 μg/ml) is only 20–50%, as previously reported [16, 38] and as shown in Fig.1.

Previously, we found that gp120-stimulated macrophages and T cells could produce several β-chemokines, including MIP-1α, MIP-1β, and RANTES [31]. The present study indicated that CRDS could completely inhibit gp120-mediated production of β-chemokines such as MIP-1α, MIP-1β, and MCP-1 production (Fig. 2 a–c), although the maximum inhibition of gp120 binding by CRDS was approximately 45% in our system (Fig. 1). CRDS also inhibited the production of these β-chemokines induced by LPS stimulation (Fig. 3). These results indicate that the inhibitory effect of CRDS on the production of MIP-1α, MIP-1β, and MCP-1 is not only related to blocking of gp120 binding but also to a direct effect on cells. On the other hand, CRDS unexpectedly enhanced the gp120-mediated RANTES production (Fig. 2 d). In addition, CRDS directly promoted the production of RANTES even in the absence of gp120 (Fig. 4). Furthermore, the production of IL-16 was increased by CRDS, regardless of the presence of gp120 (Fig. 5). The mechanism by which CRDS inhibits production of MIP-1α, MIP-1β, and MCP-1, while enhancing RANTES and IL-16 production is still unknown, and these effects cannot be explained solely by the influence of CRDS on virus attachment to CD4. We have reported previously that CRDS could inhibit not only gp120- but also LPS-mediated TNF-α production, which is known to facilitate HIV-1 replication through the induction of a transcriptional factor (NF-κB, a DNA-binding protein which binds to viral enhancer sites) in HIV-infected cells [33], and suggested that this might be important in the inhibition of HIV-1 infection in vitro [38]. Such an action may also be mainly dependent on a direct effect of CRDS rather than inhibition of gp120 binding to CD4 [38]. Furthermore, a previous study showed that CRDS could inhibit TNF-α production by LPS-injected mice [27]. Taken together, these findings suggest that CRDS has some effect on signal transduction which is concerned with regulation of the genes for chemokine/cytokine production. We cannot neglect the possibility that our findings resulted from the interaction of chemokines and cytokines produced by CRDS, which can influence each other's production [17, 22].

RANTES and IL-16, as well as MIP-1α, MIP-1β, have been reported to inhibit HIV infection in vitro. The effect of the former is mediated by competitive inhibition for cell surface receptors between RANTES and UIV [7, 9, 10], while that of IL-16 is possibly due to blocking the expression of HIV-1 proviral messenger RNA [40]. In contrast, MCP-1 has been reported to enhance HIV-1 infection in vitro [5, 34]. In addition, Bisset et al. [4] reported that the serum level of RANTES increases and that of MCP-1 decreases with reduction of the viral load by protease inhibitor therapy in HIV-1-infected patients. Our data suggest

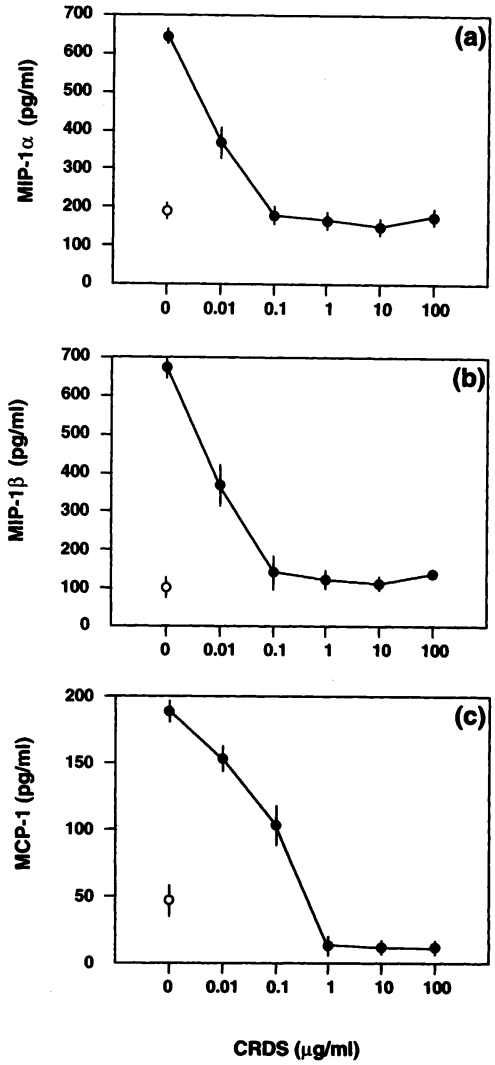

Fig. 3 Effect of CRDS on the production of β-chemokines (**a** MIP-1α; **b** MIP-1β; **c** MCP-1) by cells cultured with (●) and without (○) LPS. There were significant differences of β-chemokine levels between cultures without CRDS and with CRDS (100 μg/ml) (P<0.01) (*LPS* lipopolysaccharide)

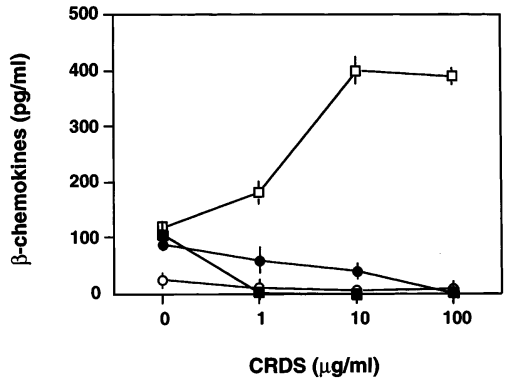

Fig. 4 Effect of CRDS on the production of RANTES (□), MIP-1α (●) , MIP-1β (○), and MCP-1 (■) by cells cultured without gp120. A significant difference in the RANTES level was found between cultures without CRDS and with CRDS (100 μg/ml) (P<0.01). There are no statistical differences of MIP-1α, MIP-1β, and MCP-1 productions between cultures without CRDS and with CRDS

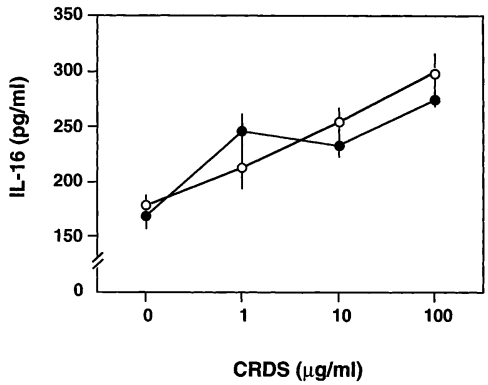

Fig. 5 Effect of CRDS on the production of IL-16 by cells incubated with (●) and without (○) gp120. A significant difference was observed in the IL-16 level between cultures without CRDS and with CRDS (100 μg/ml) (P<0.01)

that regulation of chemokines by CRDS (i.e., increase of RANTES and IL-16, and decrease of MCP-1) is significant in the inhibition of HIV-1 infection, in addition to blocking the attachment of HIV envelope glycoprotein to CD4 molecules on the cell surfaces.

Since TNF-α has been reported to promote not only viral replication but also HIV-related cachexia and gp120-mediated cell dysfunction, including down-modulation of CD4 expression, T cell activation, T cell anergy, apoptotic T cell death, and polyclonal B cell activation [19, 23–25, 28, 36, 37], the blocking of TNF-α by CRDS may help to inhibit such HIV-related clinical manifestations and immune dysregulation. Some reports have suggested that β-chemokines could induce T cell activation in vitro, although their precise role in HIV-related immune dysregulation is still unknown [30, 39]. The inhibitory effect of CRDS on production of β-chemokines (such as MIP-1α, MIP-1β, and MCP-1) may contribute to blocking of gp120-induced immune dysregulation. We are now investigating the role of CRDS in the production of other chemokines/cytokines and their effect on gp120-mediated immune dysregulation, in addition to determining which cell populations are mainly involved.

In conclusion, the regulation of chemokines/cytokine production by CRDS may be significant for its inhibition of HIV infection in addition to the blocking of inhibition of viral attachment, and this agent may potentially be useful for the prevention of HIV-1 infection.

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