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Inhibition of HIV-1 infection of peripheral blood mononuclear cells by a monoclonal antibody that binds to phosphoinositides and induces secretion of β-chemokines

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ABSTRACT

A murine IgG mAb, WR321, selected for the ability to bind to phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate, but an inability to bind to any of 17 other lipids, including phosphatidylinositol, was examined as a probe for studying interactions of HIV-1 with primary human peripheral blood mononuclear cells. The WR321 mAb broadly neutralized CCR5-tropic strains of HIV-1 to prevent infection of the cells. The mAb also exhibited direct interaction with cells in the culture, resulting in secretion of chemokines that interfered with the interaction of HIV-1 virions with CCR5, the coreceptor for HIV-1 on the susceptible cells, leading to inhibition of infection by HIV-1. Phosphoinositides that are recognized by WR321 do not exist on the external surface of cells, but are concentrated on the inner surface (cytoplasmic leaflet) of the plasma membrane. Murine anti-phosphoinositide mAbs similar to WR321 have previously been directly microinjected into a variety of cultured cells, resulting in important changes in the functions of the cells. The present results suggest that binding of a mAb to phosphoinositides, resulting in secretion of β-chemokines into the culture medium and neutralization of infection by CCR5-tropic HIV-1 of nearby susceptible cells, occurred by uptake and binding of the mAb at an intracellular location in the cultured cells that then led to secretion of HIV-1-inhibitory β-chemokines.

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1. Introduction

Antibody-based approaches to studies of the immunobiology of HIV-1, and to development of vaccines to HIV-1/AIDS, often rely on antibody-mediated inhibition (neutralization) of infection of susceptible cells. One such assay utilizes human peripheral blood mononuclear cells (PBMC) as targets for infection by HIV-1 [1]. In the course of examining the effects of monoclonal antibodies (mAbs) in the PBMC assay, we discovered that two murine IgM mAbs (PIP1 and PIP4, now known as WR301 and WR304) that were initially selected for the ability to bind to phosphatidylinositol-4-phosphate (PIP), each neutralized HIV-1 [2,3]. In addition, we discovered that an important, but rare, broadly neutralizing human IgG mAb, known as 4E10, that had previously been reported to bind simultaneously both to a linear site on the membrane proximal external region (mper) of gp41 from HIV-1 and to cardiolipin (diphosphatidylglycerol), also bound to PIP [2,4]. Despite the lipid binding similarities between 4E10 and WR304, it was subsequently found that each mAb also bound to a wide variety of other types of lipids, including phosphatidylinositol-4,5-bisphosphate (PIP2) and many other phospholipids, cholesterol, glycolipids (4E10, but not WR304, bound to glucosyl ceramide and galactosyl ceramide), a sulfated glycolipid (sulfogalactosyl ceramide, also known as sulfatide), and even a glycophospholipid (lipid A, the lipid moiety of lipopolysaccharide that covers the surface of Gram negative bacteria) [5].

Phosphoinositides comprise a group of seven intracellular phospholipid species that are reversibly generated by phosphorylation of the precursor molecule, phosphatidylinositol (PI), at positions 3,4, and 5 of the inositol ring [6]. Although PIP and PIP2 occur on plasma membranes of cells, and although PIP2 may constitute as much as 5% or more of the total lipids of plasma membranes, all of the phosphoinositides are thought to occur exclusively on the inner (cytoplasmic) leaflets of plasma membranes and on intracellular organelles where they serve as intermediates in numerous types of intracellular signaling events [6]. Despite the absence of phosphoinositides on cell surfaces, they still remained as candidates for binding of the WR304 and 4E10 mAbs to HIV-1 because

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phosphoinositides are known to be important chemical constituents of HIV-1 virions [7]. This is because phosphoinositides are functionally active both during the intracellular assembly of virions in host cells and localization of gag to the plasma membrane whereby PIP2 serves as a bridge between the matrix protein and the envelope of HIV-1 [8–11]. It was thus concluded that although both 4E10 and WR304 could neutralize HIV-1 in the PBMC assay without binding to any HIV-1 proteins, it seemed likely that the mAbs could bind to one or more HIV-1 lipids, even if the exact lipid binding sites, whether phosphoinositides or other lipids, remained unclear because of very broad lipid binding specificities of the mAbs.

Recently, a novel alternative neutralizing mechanism for anti-lipid mAbs has been proposed that does not require any direct binding of the anti-phospholipid antibodies to HIV-1. A neutralizing human mAb, CL1, was described as having binding capabilities for both cardiolipin and phosphatidylserine, but no binding to HIV-1 envelope protein, and it inhibited proliferation of CCR5-tropic, but not CXCR4-tropic HIV-1 strains in a PBMC assay [12]. In this case, release of several β-chemokines, including MIP-1α and MIP-1β, was observed in the PBMC culture fluid. β-Chemokines are believed to be effectors secreted by many cells, including CD4+ and CD8+ lymphocytes, NK cells, and monocytes in the PBMC that inhibit infection by binding to CCR5, thus blocking infection with HIV-1 strains that utilize CCR5, but not those that utilize CXCR4, as a co-receptor [13]. Based on binding of CL1 to cells in the PBMC culture, it was hypothesized that CL1 bound to plasma membrane phospholipid(s) and caused secretion of β-chemokines that interfered with binding of HIV-1 to CCR5-dependent, but not to CXCR4-dependent, viruses [12]. In the present study, we explore further the lipid binding specificities of CL1, including binding to phosphoinositides, and we describe a new murine neutralizing IgG mAb (WR321) which binds only to phosphoinositides PIP and PIP2 among 19 lipids tested, but which also causes release of HIV-1-inhibitory β-chemokines by PBMC even in the absence of HIV-1.

2. Materials and methods

2.1. Lipids, liposomes, proteins, and peptides

Recombinant, truncated HIV-1 gp41 (HXB2) (Swiss-Prot Accession Number P04578) containing amino acids 541–682 with 6 His attached to the carboxy terminus and expressed *Pichia pastoris*, was purchased from The Biotech Source (Franklin, MA). Mper23, LELDKWASLWNWFDITNWLWYIK, was synthesized in-house using FMOC chemistry and standard solid-phase techniques with free amino termini [14]. Lipids and lipid antigens were obtained as described before [5]. Multilamellar liposomes containing DMPC:Chol:PIP (1:1.5:1) were prepared at a concentration of 50 mM phospholipid containing lipid A and mper23 at 200 μg/ml [3,15].

2.2. Monoclonal antibodies

Female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were immunized intraperitoneally 6 times with 0.1 ml of liposomes at 3 week intervals. Three weeks after the last immunization, a mouse was boosted by the intravenous route and the spleen was removed 4 days after boosting. The spleen cells were fused with SP2/0 cells obtained from the American Type Culture Collection (ATCC), Chantilly, VA, and hybridomas were cloned using ClonaCell®-HY Hybridoma Kit (Stemcell Technologies, (Vancouver, BC, Canada). WR321 was grown in serum-free media (Invitrogen, Carlsbad, CA), and purified using a protein G kit. The isotype of WR321 was determined as IgG2b with a kappa light chain using an isotype kit (Roche, Diagnostics Corp., Indianapolis, IN). TFTB-1 cells, which secrete a mouse IgG to the ricin B chain, were purchased from ATCC, and the mAb was grown and purified as described above. The human lgG anti-phospholipid mAb CL1 was kindly supplied as a gift by Drs. Pojen Chen (UCLA) and Barton Haynes (Duke University). Each of the WR321 and CL1 mAbs had very low levels of endotoxin.

2.3. ELISA

Individual lipids as indicated, gp41, and mper23 peptide, were each used as capture antigens, and assays using the antigens were performed as described for mouse antibodies [3] and human antibodies [5]. Assay background was determined by using wells lacking antigen, and values less than twice the background are considered negative. Values greater than twice background are indicated by asterisks in Fig. 1. Cell culture supernatant chemokines, MIP-1α, MIP-1β, and Rantes, and interferon-γ were quantified with ELISA kits (R&D Systems, Minneapolis, MN).

2.4. HIV-1 neutralization assay

PBMC neutralization assays were performed as previously described with replication component, *Renila reniformis* luciferase

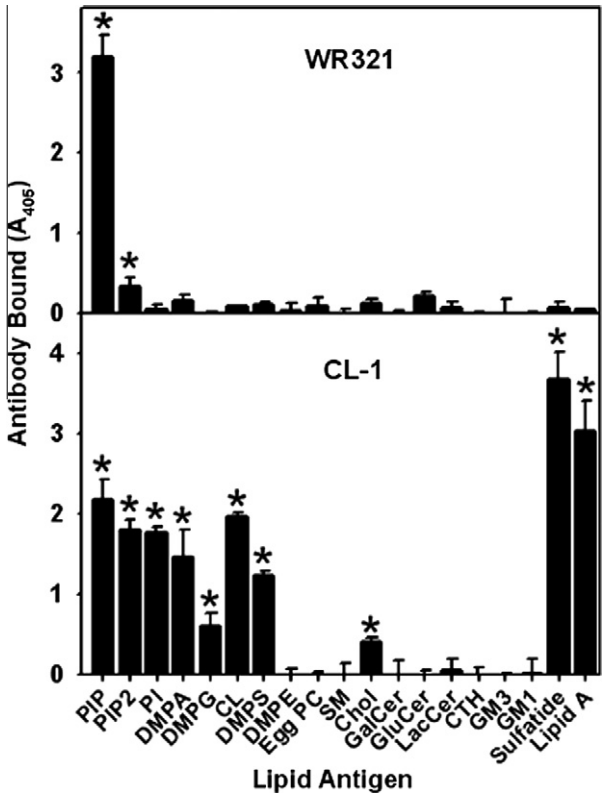

Fig. 1. Lipid binding specificity of WR321 and CL1 as measured by ELISA. Data are triplicate determination ± standard deviation of a representative assay which was repeated 3 times. Values are the absorbance of the binding seen from 100 ng of mAb to 1 nmol of coating lipid. Data have the background from wells lacking mAb subtracted. Bars with asterisks represent absorbances that were >twice background and represented positive binding. Abbreviations used were phosphatidylinositiol-4-phosphate (PIP), phosphatidylinositol-4,5-phosphate3 (PIP2), phosphatidylinositol (PI), dimyristoyl phosphatidic acid (DMPA), dimyristoyl phosphatidylglycerol (DMPG), cardiolipin (CL), dimyristoyl phosphatidylserine (DMPS), dimyristoyl phosphatidylethanolamine (DMPE), phosphatidylcholine isolated from eggs (Egg PC), sphingomyelin (SM), cholesterol (Chol), galactosylceramide (GalCer), glycosylceramide (GluCer), lactosylceramide (LacCer), ceramide trihexoside (CTH), GM3, GM1, sulfogalactosylceramide (sulfatide).

(LucR)-expressing HIV-1 reporter virus NL-LucR [3,16]. The clade B viruses, Bal and SF162, in T2A-ENV.ectoviruses were kindly provided by Christiana Ochsenbauer and John Kappes, University of Alabama at Birmingham. Viral envelope sequences from CRF01_AE CM235 and Clade C 16–29 were also cloned into LucR. T2A and are designated 01-pCM235-2-LucR. T2A (CM235) and C-ETH2220-llB-LucR (16–29), respectively [17].

3. Results

3.1. Binding specificities of WR321 and CL1 to lipids

Representative experiments showing the relative binding of WR321 and CL1 to 19 different lipids as determined by ELISA is shown in Fig. 1. CL1 bound to 7 of the 10 phospholipids (PIP, PIP2, PI, PA, PG, CL, and PS); to cholesterol; to a sulfoglycolipid (sulfatide), but not to any other glycolipid; and it bound to lipid A. In contrast, WR321 bound only to PIP and to a lesser degree to PIP2. Neither of the mAbs bound to gp41 from HIV-1 or the mper peptide derived from gp41 (data not shown). These data confirm the anti-CL and anti-PS binding properties of CL1 [12], but also demonstrate that the lipid binding specificity of CL1 is relatively broad and is similar to the relatively broad lipid binding specificities of the previously reported murine mAb WR304 [5]. In contrast, we conclude that phosphoinositides represent the dominant, and probably the sole lipid binding specificities, of WR321.

3.2. Neutralization of HIV-1 by WR321 and CL1 for prevention of infection of PBMC

The potent broad neutralization properties of CL1 reported by Moody et al. [12] were confirmed with four different HIV-1 viruses in four representative experiments with four different PBMC donors (Fig. 2A–D). Similarly, WR321 also exhibited neutralization with each virus, but the neutralizing titers were lower than those of CL1. The relative potencies of WR321 and CL1 for 50% neutralization (IC_{50}) of each of the viruses derived from 2 to 4 experiments is shown in Table 1. The negative control murine IgG mAb, TFTB, failed to exhibit significant neutralization.

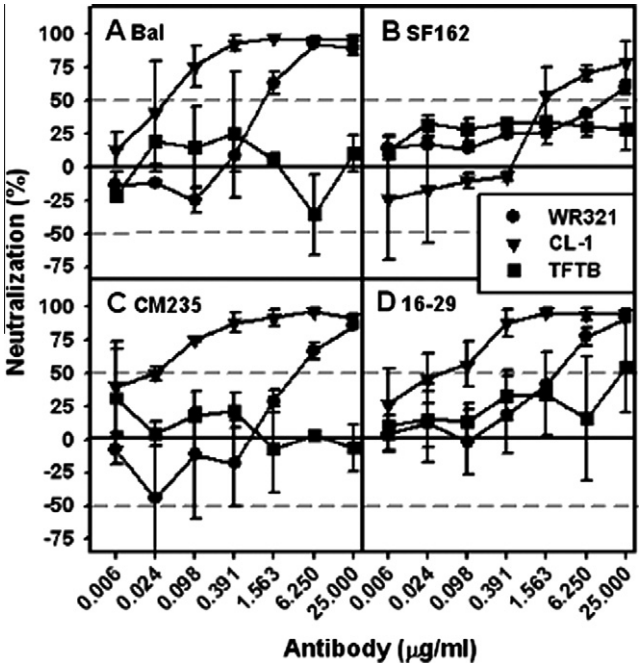

Fig. 2. Neutralization of HIV-1 by WR321 and CL1. MAbs were mixed with four different Renila luciferase constructs of HIV-1 and the mixture was added to PHA-stimulated PMBC. Four days later, virus production was measured. Values shown are the means ± standard deviation of duplicate samples run by two different operators from representative experiments (See Table 1 for number of experiments). Dashed lines represent 50% neutralization or 50% enhancement.

3.3. Chemokine secretion elicited from PBMC by WR321 and CL1

As shown in Fig. 3, with two different PBMC donors, WR321 and CL1 each caused considerable secretion of the β-chemokines MIP-lα and MIP-lβ. As indicated, the secretion by PBMC induced by WR321 occurred either in the presence or absence of HIV-1. The secretion of both of these cytokines by PBMC in the presence of WR321, as shown in Fig. 3, suggests that the secretion of the β-chemokines was partly, or possibly even completely, due to binding of the both the CL1 and WR321 mAbs to phosphoinositides. The data thus further suggest that phosphoinositides on (or in) the PBMC may be readily available for binding of antibodies that are highly specific for phosphoinositides.

4. Discussion

The murine mAbs WR301 and WR304 were originally created after immunization with liposomes containing PIP and lipid A, and the mAbs were then selected for binding and complement-dependent damage to liposomes containing PIP but not to liposomes lacking PIP [18]. This general method has also been employed by others for producing and/or selecting mAbs to phosphoinositides [19,20]. However, it is now apparent that considerable unexpected cross-reactivity of antibodies produced by this method can occur with other lipids [5,21]. Despite this, the observations in the present work of the lack of cross-reactivity of WR321 with other lipids, or with peptide or protein, and previous observations with similar anti-phosphoinositide mAbs [22], demonstrate that exquisite specificities of murine mAbs for phosphoinositides can be achieved. The present work further demonstrates that this can occur even when the liposomes used for immunization also contain a peptide antigen in addition to the phosphoinositide.

Based on the narrow specific binding characteristics of WR321, it appears reasonable to conclude that all of the observed secretion of β-chemokines in this study could have occurred in response to binding of the WR321 to one or more phosphoinositides, including PIP or PIP2. However, in view of the secretion of β-chemokines after incubation of intact cells with WR321 in the absence of HIV-1, this conclusion presents an interesting conundrum because of the complete absence of phosphoinositides on the outer surface of plasma membranes of non-permeabilized cells. In view of this, we propose the hypothesis that WR321 could have entered one or more of the cell types in the PBMC population, either by endocytosis, pinocytosis, or macropinocytosis, where it then exerted intracellular binding effects on the inner leaflet of plasma membranes or other intracellular organelles, resulting in the secretion of β-chemokines.

Table 1
Neutralization of four different strains of HIV-1.

Virus	Mean IC_{50} (μg/ml) ± SD[a]		
	WR321	CL-1	TFTB
SF162	2.05 ± 1.24 (*N* = 3)*	0.35 ± 0.19 (*N* = 3)	>25 (*N* = 2)
Bal	3.32 ± 2.27 (*N* = 4)	0.29 ± 0.10 (*N* = 3)	>25 (*N* = 3)
16/19	1.79 ± 0.22 (*N* = 3)	0.24 ± 0.22 (*N* = 3)	>25 (*N* = 2)
CM235	4.33 ± 1.52 (*N* = 3)	0.26 ± 0.06 (*N* = 2)	>25 (*N* = 3)

[a] IC_{50} was calculated from the antibody dose response curves as illustrated in Fig. 2. Assays were conducted in duplicate by two different operators. Values are mean IC_{50} of the number of independent experiments indicated.

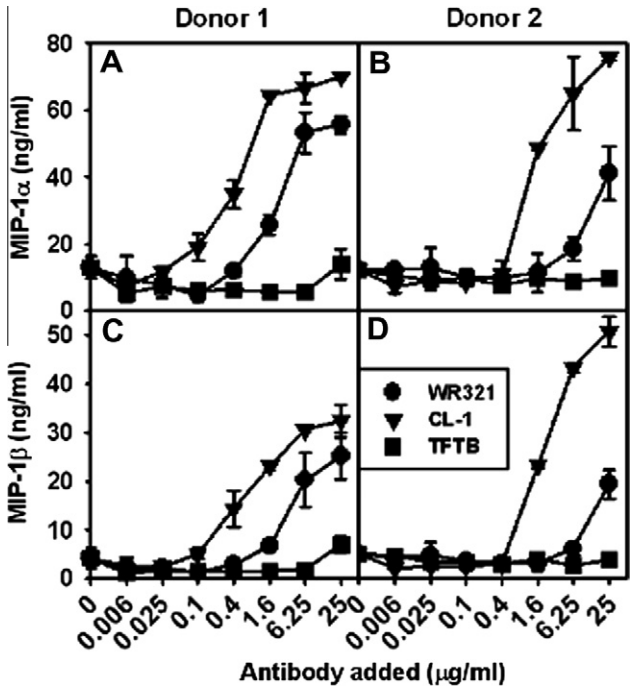

Fig. 3. Chemokine secretion induced by WR321 and CL1. MAbs were mixed with HIV-1 (Bal strain) and added to PHA stimulated PBMC. After 4 days, the chemokines were measured in the culture supernatant. Data are from two different donors for MIP-1α (A and B) and MIP-1β (C and D). WR321 did not induce secretion of Rantes or interferon-γ, and only low levels of secretion of these latter substances were induced by CL1 (data not shown). Similar findings were observed when the mAbs were incubated with PBMC without virus. Values shown are means ± standard deviation of two determinations.

The exact intracellular binding site of WR321, if it does occur, will require further detailed intracellular imaging of the trafficking pattern of the mAb. Furthermore, PIP and PIP2 represent only two of the seven intracellular phosphoinositide species, and further analysis of binding specificities could reveal binding to one or more of the other phosphoinositide species. However, regardless of which intracellular phosphoinoside species is involved, this hypothesis would represent a novel HIV-1 neutralization mechanism by which intracellular binding of ingested antibodies exerts an extracellular inhibitory effect on HIV-1 infection of nearby cells.

In support of this hypothesis of possible intracellular effects of WR321 exerted through binding to one or more intracellular membrane phosphoinositides, it is known that phosphoinositides, especially PIP2 and PIP, are present in relatively high concentrations on the inner leaflet of the plasma membrane lipid bilayer and on other intracellular organelles [6]. In addition, mAbs to PIP2 that were directly microinjected into the cytoplasms of individual non-permeabilized cultured cells reportedly bound to intracellular PIP2, thereby leading to a variety of important physiological effects in various cells [20,22–26]. Although the exact intracellular trafficking patterns and intracellular binding sites of ingested mAbs to phosphoinositides are unknown, the previous studies with microinjected mAbs to phosphoinositides that were similar to WR321 suggest that such uptake might have important biological effects. The exact mechanism(s) by which ingestion and intracellular binding of our anti-PIP mAb might have caused the secretion of β-chemokines is also not yet clear. However, we suggest the possibility that the cytoplasmic uptake of a mAb to phosphoinositides might have been a threatening event to the cell that could have represented a unique type of intracellular "danger signal". The secretion of β-chemokines induced by the mAb might then have been a reaction that was similar to other types of mechanisms linking chemokine secretion to innate and adaptive immunity [27].

It should also be pointed out that plus-strand RNA virus families (including picornoviruses, coronaviruses, and flaviviruses) are known to cause remodeling of intracellular organelles of infected host cells to promote PIP lipid-enriched uncoated membranes that are essential for viral RNA replication [28]. In view of the present work, it is theoretically possible that cytoplasmic uptake of antibodies that bind to PIP or other phosphoinositides might also have an impact on the intracellular replication of plus-strand RNA viruses.

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