RAPID COMMUNICATION

Inhibition of Productive Human Immunodeficiency Virus-1 Infection by Cobalamins

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Various cobalamins act as important enzyme cofactors and modulate cellular function. We investigated cobalamins for their abilities to modify productive human immunodeficiency virus-1 (HIV-1) infection of hematopoietic cells in vitro. We show that hydroxocobalamin (OH-Cbl), methylcobalamin (Me-Cbl), and adenosylcobalamin (Ado-Cbl) inhibit HIV-1 infection of normal human monocytes and lymphocytes. The inhibitory effects were noted when analyzing the monocytotrophic strains HIV-1-Bal and HIV-1-ADA as well as the lymphocytotrophic strain HIV-1-LAI.

Cobalamins did not modify binding of gp120 to CD4 or block early steps in viral life cycle, inhibit reverse transcriptase, inhibit induction of HIV-1 expression from cells with established or latent infection, or modify monocyte interferon-α production. Because of the ability to achieve high blood and tissue levels of cobalamins in vivo and the general lack of toxicity, cobalamins should be considered as potentially useful agents for the treatment of HIV-1 infection. This is a US government work. There are no restrictions on its use.

PRODUCTIVE CELLULAR infection with human immunodeficiency virus-1 (HIV-1) is controlled by several different viral and host cell factors. Recent work by other investigators has shown that heme and certain porphyrins can inhibit HIV-1 infection of cells in vitro and that the effects are apparently mediated by inhibition of viral envelope-cellular CD4 binding. Because of the structural similarities of heme and cobalamins, we sought to determine if cobalamins could modify HIV-1 infection of cells in vitro. We note in the series of experiments reported here that various cobalamin derivatives inhibit the ability of HIV-1 to cause productive infection of human monocytes (Mo) and normal blood lymphocytes (peripheral blood mononuclear cells [PBLC]).

MATERIALS AND METHODS

Cells. Monocytes and PBL were isolated and cultured as noted before. Monocytes were isolated by sequential ficoll-sodium diatrizoate and Percoll gradients with subsequent adherence to cultureware plastic and then cultured in Dulbecco's modified Eagle medium (GIBCO, Grand Island, NY) with 10% unheated human serum. Mitogen-simulated PBL were prepared as noted before using anti-CD3 and anti-CD28 antibodies with interleukin-2 (IL-2).

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VIRUSES, VIRAL REVERSE TRANSCRIPTASE (RT) ASSAY, GP120-C4D BINDING ASSAY, P24 ASSAY, POLYMERASE CHAIN REACTION (PCR) ASSAYS, AND INTERFERON-α ASSAYS. The monocytotropic strain HIV-1-Bal and the lymphocytotropic strain HIV-1-LAI are maintained in our laboratory as noted before. RT was measured as described before using a phosphoimager device for quantification. Binding of recombinant gp120 to recombinant soluble CD4 was performed in microtiter plates using reagents from Genetech (South San Francisco, CA). In brief, wells were first coated with 200 ng of gp120 and CD4 (40 ng in 0.1 mL of phosphate-buffered saline [PBS]) was added with varying amounts of the designated cobalamins to allow binding. Bound CD4 was then detected using a horseradish peroxidase-conjugated anti-CD4 monoclonal antibody. HIV-1 p24 antigen was measured using a capture enzyme-linked immunosorbent assay (ELISA) method (Coulter, Inc, Hialeah, FL). The PCR-based assay for newly synthesized viral DNA was performed as noted before.

In brief, cells were inoculated with DNAse-treated virus alone or in the presence of the appropriate cobalamin. Cells were then washed and cultured in medium for 20 hours with 10 μg/mL anti-CD4 antibody (Leu3A from Becton Dickinson, Mountain View, CA) to prevent any further viral entry. This time period simulates a single round of virus infection. Cellular lysates were prepared, and total cellular DNA was subjected to 38 cycles of PCR amplification using primers that flank the nef gene to generate an approximately 1.2-kb HIV-1-specific PCR product. β-Globin-specific primers were used to assure approximate equal numbers of cells in the samples. IFN-α assays were performed using the specific enzyme-linked immunosorbent assay (ELISA) BioSource International (Camarillo, CA), an assay that can detect IFN-α down to a minimum of 10 pg/mL.

Cobalamins. Cobalamins were from Sigma Chemical Co (St Louis, MO) or ICN (Costa Mesa, CA); cobalamins from either source gave comparable results. The cobalamins had extremely low endotoxin content, as determined by Limulus amebocyte lysate testing (Whittaker Bioproducts, Walkersville, MD). When tested as a 3 mmol/L solution, the cobalamins had less than 0.25 EU/mL.

RESULTS

OH-Cbl, Me-Cbl, Ado-Cbl, and cyanocobalamin (CN-Cbl) were not toxic for Mo, PBL, or cells of the human leukemia cell lines Jurkat, CEM, IL-60, or U937 at concentrations ≤1,000 μmol/L (Fig 1). When added to the cultures of monocytes or PBL at the time of viral inoculation and left in throughout the culture period, there was potent inhibition of productive infection as judged by supernatant medium RT

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Submitted April 24, 1995; accepted June 1, 1995.

Supported in part by the VA Research Service, the Durham VA Research Center for AIDS and HIV infections, the James R. Swiger Hematology Research Fund, and National Institutes of Health Award No. AR-39162.

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0006-4971/95/8604-0051$00.00/0
Fig 1. Effect of CN-Cbl, OH-Cbl, Me-Cbl, and Ado-Cbl on HIV-1 infection of monocytes, lymphocytes, and hematopoietic cell line cells. (A) Effects of OH-Cbl on HIV-1-BaL infection of monocytes. Monocytes were inoculated on the day of isolation with HIV-1-BaL (MOI ~ 0.001) and cultured with OH-Cbl for 14 days. Supernatant medium RT was then measured. Results are displayed as fraction of control RT activity (mean ± SEM of triplicate samples). (B) Effects of CN-Cbl, OH-Cbl, and Ado-Cbl on HIV-1-BaL infection of monocytes. Monocytes were inoculated on the day of isolation with HIV-1-BaL (MOI ~ 0.001) and cultured with the designated cobalamin for 14 days. Supernatant medium RT was then measured. In results not shown, Me-Cbl had inhibitory actions comparable to that of OH-Cbl and Ado-Cbl. Comparable patterns of inhibition were seen with HIV-1-ADA (data not shown). Results are displayed as fraction of control RT activity (mean ± SEM of triplicate samples). (C) Effects of CN-Cbl, OH-Cbl, Me-Cbl, and Ado-Cbl on HIV-1-LAI infection of PBL. PBL were inoculated with HIV-1-LAI (MOI ~ 0.001) and cultured for with the designated cobalamin for 14 days. Fresh media was added every 2 days. RT activity in supernatant medium sampled on day 14 was measured. Results are displayed as fraction of control RT activity (mean ± SEM of triplicates). (D) Effects of OH-Cbl on HIV-1-LAI infection of leukemia cell line cells. Cells of the T-lymphoblast cell lines Jurkat and CEM, the myeloblast cell line HL-60, and the monoblast cell line U937 were inoculated with HIV-1-LAI (MOI ~ 0.001) and cultured with OH-Cbl for 8 days. Supernatant medium RT was then measured. Results are displayed as fraction of control RT activity (mean ± SEM of triplicates). Cobalamins were not toxic for Mo, lymphocytes, and leukemia cell line cells as determined by morphology under phase microscopy, Wright staining of methanol-fixed cells, trypan blue exclusion, and thymidine incorporation (PBL and cell line cells) (data not shown).

activity and the formation of monocyte syncytia (multinucleated giant cells; Figs 1A, B, C and 2). The concentration required for 50% inhibition (IC_{50}) was approximately 2 to 4 μmol/L when assessed in monocytes and 25 to 50 μmol/L in normal PBL. CN-Cbl exhibited little or no inhibitory activity in all experiments using monocytes and PBL. If we assayed supernatant medium p24 antigen instead of RT as a measurement of viral infection in monocytes, OH-Cbl caused a comparable inhibition (data not shown). Me-Cbl and Ado-Cbl (but not CN-Cbl and OH-Cbl) are light-sensitive and are rapidly converted primarily to OH-Cbl. Thus, it is likely that in our experiments (which were performed under reduced light, but...
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Fig 2. Effects of OH-Cbl on multinucleated giant cell (syncytium) formation in monocytes inoculated with HIV-1-BaL. Monocytes were inoculated with HIV-1-BaL (MOI ~0.001) and cultured with or without 34 μmol/L OH-Cbl or 500 μmol/L CN-Cbl for 14 days. The cells were then rinsed with saline, fixed with 100% methanol, and stained with Wright-Giemsa stain. Populations of monocytes not inoculated with virus (A, C, and E) are generally mononuclear, whereas those inoculated with HIV-1-BaL cultured alone (B) or with CN-Cbl (F) have numerous multinucleated giant cells. Populations of HIV-1-inoculated monocytes cultured with OH-Cbl (D) do not have multinucleated giant cells. Comparable effects were noted in doses ranging from 34 to 1,000 μmol/L using each cobalamin. (Wright stain; original magnification x 200.)

not absolute darkness) significant fractions of Me-Cbl and Ado-Cbl were converted to OH-Cbl in vitro.

Although OH-Cbl, Me-Cbl, and Ado-Cbl inhibited HIV-1 infection of these primary, normal leukocytes, they had much less inhibitory effect on the infection of cells of the human T lymphoblast lines Jurkat and CEM, the myeloblastic line HL-60, or the monocytic-like cell line U937 (Fig 1D). The inhibition of productive infection was not strain specific—OH-Cbl, Me-Cbl, and Ado-Cbl inhibited infection of monocytes with the monocytotropic strains HIV-1-BaL or HIV-1-ADA or of PBL with HIV-1-BaL or the lymphocytic strain HIV-1-LAI. The cobalamins did not inhibit RT activity. If we added 1 to 1,000 μmol/L CN-Cbl, OH-Cbl, Me-Cbl, or Ado-Cbl to cell-free medium containing RT, none of the cobalamins modified RT activity (data not shown).

If monocytes were pretreated with 250 μmol/L OH-Cbl for 24 hours, washed well, and then inoculated with HIV-1-BaL, the OH-Cbl-treated cells were protected against HIV-1 infection (Fig 3A). However, the degree of inhibition was less than that in cultures which OH-Cbl was present throughout the culture period. If monocytes were incubated simultaneously with 250 μmol/L OH-Cbl and HIV-1 for 4 hours, washed, and then cultured for 10 days, OH-Cbl-treated cells expressed less viral RT (Fig 3B). When monocytes were inoculated with HIV-1 and cultured for 6 days (a time at which they displayed multinucleated giant cell formation and RT production), washed thoroughly, and then cultured for 10 more days with 250 μmol/L OH-Cbl, there was no inhibition of their subsequent production of RT (Fig 3C). To determine if OH-Cbl would inhibit the ability of latently infected leukemia cells to produce RT after stimulation with tumor necrosis factor (TNF), we treated OM-10.1 cells (an HL-60 cell line with stably integrated proviral HIV-1) with 500 U/mL TNF (with varying amounts of OH-Cbl). OH-Cbl caused only slight decreases in the expression of the induced viral RT (Fig 3D). The inability of the cobalamins to effectively inhibit HIV-1 production in previously infected monocytes and in latently infected leukemia cells is similar to that seen when using drugs such as the RT inhibitors zidovudine, didanosine, and zalcitabine.

HIV-1 binds to cells principally via interaction with cell membrane CD4. Other investigators have shown that heme and various porphyrins inhibit HIV-1 infection and that this inhibition generally parallels the compounds' abilities to block gp120-CD4 binding. To determine if cobalamins modified the binding of HIV-1 gp120 to CD4, we tested the ability of CN-Cbl, OH-Cbl, Me-Cbl, or Ado-Cbl to block binding of recombinant CD4 with gp120 from HIV-1-LAI or HIV-1-MN (Fig 4A and B). None of the cobalamins inhibited binding. Likewise, neither CN-Cbl nor OH-Cbl inhibited early steps in the life cycle of HIV-1-BaL in monocytes (viral-cell fusion, viral entry, and reverse transcription; Fig 4C).

It was possible that the cobalamins induced an intrinsic HIV-1 resistance system (eg, induction of IFN-α) within the cells. To determine if cobalamins might induce cellular production of IFN-α (which could then render cells resistant to HIV-1 infection), we treated monocytes with 15 to 500 μmol/L CN-Cbl or OH-Cbl for 3 days and measured IFN-α in the supernatant media using a sensitive ELISA. IFN-α production was not induced by the cobalamin treatments; eg, the supernatant fluid of monocytes cultured 3 days in media with 10% human serum contained 71 ± 1 pg/mL IFN-α, whereas that of monocytes cultured in media with 10% human serum and 500 μmol/L OH-Cbl contained 74 ± 5 pg/mL and that of monocytes cultured in media with 10% human serum and 500 μmol/L CN-Cbl contained 71 ± 1 pg/mL. Likewise, monocytes inoculated with HIV-
**Fig 3.** Effects of OH-Cbl on HIV-1 productive infection by pretreatment of cells with OH-Cbl before inoculation, by coincubation of OH-Cbl with virus during inoculation, by treatment of previously infected monocytes, or by treatment of latently infected myeloid cells. (A) Inhibition of HIV-1 infection of monocytes by OH-Cbl pretreatment. Monocytes were treated for 24 hours with varying amounts of OH-Cbl. The cells were then washed twice with PBS and then inoculated with HIV-1-BaL (MOI -0.001) and cultured for 14 days. Supernatant medium RT activity was then measured. Results are displayed as fraction of control RT activity (mean ± SEM of triplicates). (B) Inhibition of HIV-1 infection of monocytes by coincubation with OH-Cbl during the viral inoculation step. Cells were incubated with HIV-1-BaL (MOI -0.001) with varying amounts of OH-Cbl for 4 hours and then washed twice with PBS to remove unbound cobalamin and virus. They were then cultured for 14 days without further addition of virus or OH-Cbl. Supernatant medium RT activity was then measured. Results are displayed as fraction of control RT activity (mean ± SEM of triplicates). (C) Lack of inhibitory effect of OH-Cbl on viral production by previously infected monocytes. Monocytes were inoculated with HIV-1-BaL (MOI -0.001) and cultured for 7 days. The cells were then twice with PBS and incubated for 7 more days with different amounts of OH-Cbl. Supernatant medium RT activity was then measured. Results are displayed as fraction of control RT activity (mean ± SEM of triplicates). (D) Effect of OH-Cbl on induction of viral production by TNF-treated, latently infected OM-10.1 cells. The cells were treated with 500 U/mL recombinant human TNF with varying amounts of OH-Cbl for 3 days. Supernatant medium RT activity was then measured. Results are displayed as fraction of control RT activity (mean ± SEM of triplicates). Control OM-10.1 cells not treated with TNF did not express any RT activity.

l-BaL cultured with or without CN-Cbl or OH-Cbl did not vary significantly in their IFN-α production (data not shown).

**DISCUSSION**

Cobalamins are polycyclic compounds containing a cobalt within the corrin ring, an upper axial ligand for the cobalt (e.g., cyano-, hydroxo-, methyl-, or adenosyl-), and a dimethylbenzimidazole nucleotide moiety that forms the lower axial cobalt ligand. Cobalamins are absorbed from the gut into the blood by processes that require the proteins intrinsic factor and transcobalamin II (TC-II). They are transported in the blood bound to TC-II; the Cbl–TC-II complex enters cells after binding to TC-II membrane receptors. CN-Cbl (vitamin B_{12}) is a relatively nonreactive, stabilized cobalamin that is not found normally in the body. It is used pharmacologically for the treatment of B_{12} deficiency states in humans. In vivo, cyanide is removed from the cobalt with production...
of OH-Cbl. OH-Cbl is converted to Me-Cbl or Ado-Cbl, and these are cofactors for enzymes including methionine synthase (Me-Cbl) and methylmalonyl CoA mutase (Ado-Cbl). Cobalamin deficiency results in neurologic and hematologic abnormalities. In comparison to CN-Cbl, OH-Cbl is better retained in the body, more bioavailable to cells, and more efficiently processed by cells in vitro to active cobalamin cofactors. This could explain in part the relative ineffectiveness of CN-Cbl as an inhibitor. The cell-specific inhibition effects (inhibition in normal monocytes and lymphocytes, but less in cell line cells) could be related to differences in TC-II receptors, with cells of some types being able to take up and accumulate more of the cobalamin. Alternatively, cells of some types might have unique abilities to convert the cobalamin to a derivative with antiviral activity. The inhibition of HIV-1 infection appears to be pretranscriptional and not related to early steps in the viral life cycle (Figs 3 and 4). Furthermore, the cobalamins do not inhibit reverse transcriptase and, unlike lipopolysaccharide, they do not increase IFN-α production by monocytes.

In addition to participation in important metabolic enzyme reactions, cobalamins may also bind nitric oxide (NO) and modify its actions in vitro and in vivo. In separate work, we have noted that the cobalt of OH-Cbl avidly binds NO.
Pietreforte et al. showed that Crease, 2h.2R, Bukrinsky et al. have noted that HIV-1 infection may cause neurologic disease. Generally, human mononuclear phagocytes can generate only low levels of NO in vitro, but after stimulation, this production may increase. Bukrinsky et al. have noted that HIV-1-infected monocytes have increased production of NO in vitro. Pienteforte et al. showed that HIV-1 gp120 causes human monocytes to produce NO. Thus, OH-Cbl could bind NO produced within or near an infected cell and by some mechanism inhibit productive HIV-1 infection. It has been noted that NO can activate NF-kB. Because activation of NF-kB can enhance HIV-1 expression, (OH-Cbl) could inhibit NO production in vitro, but after stimulation, this production may increase. OH-Cbl quenching of NO could inhibit NO-kb activation and reduce HIV-1 expression.

An appreciable percentage of individuals with HIV-1 infection have been noted to have low blood levels of vitamin B12, but not all show clinical evidence of vitamin B12 deficiency. Aspects of neurologic disorders and brain pathology seen in those with HIV-1 infection have similarities to those noted in vitamin B12-deficient patients (not infected with HIV-1) with neurologic disease. Dawson et al. have found that brain cells may overproduce NO during HIV-1 infection or to gp120 and that the locally produced NO might be directly cytotoxic to neural cells. Individuals with HIV-1 infection and central nervous system (CNS) abnormalities have been noted to have disturbances in their CNS methyl group metabolism, as judged by cerebrospinal fluid studies, indicating a possible functional CNS deficiency of cobalamins. Also, restoration of cobalamin levels in humans appears to improve information processing in HIV-1-infected subjects. Me-Cbl has been noted to protect against NO-mediated neuronal toxicity in vitro. It is possible that locoregional neuro NO production in the nervous system could serve to modify cobalamin levels in humans are generally nontoxic. OH-Cbl has been administered in very high doses to humans for treatment of cyanide poisoning. Noted side effects are minimal and include red-colored urine and transient high blood pressure (presumably because of OH-Cbl binding and quenching of NO). Based on our in vitro findings, we propose that experimental treatment of HIV-1-infected patients with high doses of OH-Cbl might (in a nontoxic fashion) inhibit HIV-1 infection in vivo.

ACKNOWLEDGMENT
We thank Dr. Thomas Matthews and Dr. Barton Haynes for helpful discussions, Teresa Greenwell for technical assistance, and the VA Medical Media Section for photographic assistance.

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