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HIV INFECTION DECREASES INTRACELLULAR NICOTINAMIDE ADENINE DINUCLEOTIDE [NAD]

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Summary We report that HIV-1 infection of human cells *in vitro* leads to significant decreases in the intracellular concentration of NAD. This decrease varies with viral load and HIV strain. In tissue culture, cells lacking CD4 receptors or cells incubated with heat inactivated virus do not demonstrate this decrease in NAD. Nicotinamide, the amide form of the vitamin niacin, increases intracellular NAD levels in uninfected cells as expected. Our data demonstrate that nicotinamide also maintains increased intracellular NAD concentrations in HIV infected cells. We conclude that HIV induces a state of intracellular pellagra which is reversed by the administration of nicotinamide.

We have demonstrated that NAm, the amide form of the vitamin niacin, inhibits HIV production in both acutely and chronically infected cells (1). This line of investigation was undertaken in light of the observation that HIV infected patients sometimes develop symptoms suggestive of pellagra (1). Pellagra, the clinical condition which develops with niacin depletion, is manifested on a cellular level by a decreased NAD, and this can be corrected with niacin supplementation (2,3). We present evidence in this paper that HIV infection decreases intracellular NAD levels, and that this depletion is reversed with supplemental NAm; a phenomena which we have called "intracellular pellagra."

MATERIALS AND METHODS

Primary Cells and Cell Lines

The cells were maintained as either suspension or monolayer cultures at 37°C with 5% CO_2. All of the media used were supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and L-glutamine (540 µg/ml). PBLs - the lymphocytes of healthy HIV seronegative donors were isolated from peripheral blood by Ficoll gradient technique. The lymphocytes were phytohemagglutinin stimulated [5µg/ml] and then maintained as a suspension culture in RPMI 1640 media containing IL2. Each experiment using PBLs was initiated on the fourth day following phlebotomy. TE 671 cells - are a human

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Abbreviations: ADP = adenosine di-phoshate; IL-2 = interleuken-2; NAD = nicotinamide adenine dinucleotide; NAm = nicotinamide; PARP = poly ADP ribose polymerase; PBLs = peripheral blood lymphocytes; PHA = phytohemagglutinin; PMA = phorbal myristate acetate.

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rhabdomyosarcoma cell line. They are maintained as a monolayer culture in Dulbecco's modified Eagle media. Recombinant retroviral constructs containing the CD4 gene were used to derive the viral particles for infecting TE671 cells. Following infection several clones were analyzed to select cell lines which optimally expressed CD4 on the cell surface. U1 cells - are a subclone of U937 cells, a human monocytoid cell line. U1 cells each contain two copies of stably integrated HIV-1. They demonstrate low level constitutive HIV-1 expression, but are capable of inducable high level HIV-1 expression by PMA.

HIV Strains
Stocks of HIV used in experiments were prepared by transfection of proviral DNA into RD cells (4). HIV-HXB2 is a cloned viral isolate known as λHXB2 (5), and HIV-Z6 is a cloned viral isolate known as pZ6neo (6). Viral stocks were quantitated and standardized using commercially available p24 antigen ELISA kits (Coulter, Inc.); virus quantities are expressed as nanogram equivalents of HIV p24 antigen. Heat inactivated virus was prepared by incubating the virus at 60°C for 1 hour following p24 quantitation.

NAD Assay
Total cellular NAD+ was quantitated following alkaline isolation by standard enzyme cycling technique (7). In brief, the cells were washed in PBS and then extracted with alkaline solution (0.1M NaOH). The solution was then neutralized with H_3PO_4 and centrifuged for 8 minutes at 3000 rpm. Alcohol dehydrogenase (Sigma) was added to a solution containing cell lysate, ethanol, and MTT(Sigma). MTT acts as both the terminal electron acceptor, and the chromophore (read at 570λ).

RESULTS

NAm increases intracellular NAD

Mammals can use nicotinic acid, nicotinamide, or tryptophan as the initial precursor for the synthesis of NAD. There is evidence for tissue specificity in the precursor usage for this biosynthesis (8). In Table I, HIV negative donor PBLs demonstrate a significant response within 24 hours to the addition of NAm to the standard culture media. The data show a greater than 4 fold increase in NAD with the addition of 5 mM NAm to the culture media, and greater than 5 fold increase with the addition of 10 mM NAm to the culture media. It should be noted that standard RPMI 1640 media (Gibco-BRL) contains 8μM nicotinamide, and fetal bovine serum contains an unquantified (likely nanomolar) amount of nicotinamide (9).

We observed a wide range of baseline NAD concentrations in the different cell types which were studied. In general, established cell lines had NAD detectable by enzyme cycling in the picomolar range per 10^6 cells, while primary cells had NAD in the

Table I. Increased NAD in PBLs maintained in NAm supplemented media

media [NAm]	intracellular [NAD]
control	60 pmoles
5 mM	248 pmoles
10 mM	332 pmoles

Uninfected PBLs incubated in RPMI 1640 media supplemented with Nicotinamide for 24 hrs. Intracellular NAD concentrations were determined by the enzyme cycling method per 10^7 cells.

picomolar range per 10^7 cells. Importantly, while cell lines demonstrated reproducible results in different experiments, PBLs from different donors had up to two fold differences in baseline NAD levels (compare controls in Table I and II). Therefore, in all experiments using PBLs we compare only to the control from the same donation.

HIV decreases intracellular NAD

Effect of Viral Load and Viral Strain on NAD

The rapidity of new virus production, and the quantity of viral progeny *in vitro* is HIV strain dependent. HIV-HXB2 and HIV-Z6 are two commonly used laboratory strains of HIV(5, 6). Both of these HIV strains can infect human PBLs, however the Z6 strain is a more aggressive virus which leads to an earlier and more pronounced burst of viral progeny. When comparing these viral strains we found that the drop in NAD was related to both virus strain and the amount of virus added to the cell culture. Table II demonstrates this effect on resultant cellular NAD in acutely infected PBLs; measurements were obtained 60 hours post infection from five simultaneously cultured wells with either HIV-HXB2 or HIV-Z6. Increased input HXB2 lead to an incremental increase in NAD depletion; equivalent amounts of Z6 and HXB2 lead to a 20% greater drop in NAD with the more aggressive virus.

Effect of Viral Binding and Entry on NAD

Productive HIV infection cannot take place in the absence of cellular viral receptors. The CD4 molecule has been demonstrated to be the primary entry molecule for HIV into cells (10). Using a cell line which does not express the CD4 molecule, and a subclone which expresses CD4 as a result of stable transfection, we report data on an experiment which investigated whether the entry of HIV particles via CD4 was needed for NAD depletion or if the depletion occurred as a result of viral particle interaction with the cell surface. In Figure 1 controls using either no virus or heat inactivated virus demonstrate less than 5% variation in NAD, however infection of CD4 positive cells with virus leads to a 28% decrease in NAD in the first 18 hours of acute infection.

Table II. Effect of HIV quantity and viral strain on intracellular NAD

HIV viral strain	Amount of virus	intracellular [NAD]
negative control	0 ng	136.0 pmoles
HXB2	50 ng	119.0 pmoles
	100 ng	94.0 pmoles
	150 ng	82.0 pmoles
Z6	100 ng	67.0 pmoles

PBLs in RPMI 1640 media were acutely infected with HIV then subject to NAD quantitation by the enzyme cycling method. The NAD quantitations were done at 60 hrs. post infection on 10^7 cells.

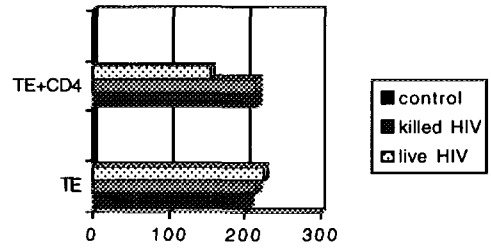

Figure 1. NAD is decreased only under conditions which support HIV infection. TE671 cells and the subclone TE671+CD4 cells exposed to either 130 ng of live HXB2 or an equivalent amount of heat inactivated virus for 18 hours. The bars compare the NAD values derived by enzyme cycling on 10^6 cells.

NAm maintains NAD in HIV infected cells

In earlier experiments, we demonstrated a dose dependent inhibition of HIV by nicotinamide in both PBLs, and in chronically infected U1 cells following stimulation with PMA(1). In this set of experiments we demonstrate that these same cell types undergo a HIV infection dependent NAD decrease, and that NAm increases the intracellular NAD despite HIV infection. In Tables III and IV the phenomenon of NAm induced prevention of NAD depletion in HIV infected cells is shown.

DISCUSSION

Our data suggests that metabolic pathways involving NAD are an important area for interaction between HIV-1 and human cells. NAD is a pyridine nucleotide which is involved in a number of normal cellular functions, including: coenzyme electron transfer (11), ADP-ribosylation of proteins (12), and the formation of cyclic ADP-ribose (13). Our results suggest that both host factors and viral factors lead to variability in the extent of NAD depletion associated with HIV infection. The data presented here provide the initial step towards understanding how NAD pathways are involved directly in the HIV-1 life cycle. Future studies will elucidate which pathways are primarily involved, and which if any are secondarily effected by decreases in available NAD.

Table III. NAm maintains elevated NAD in PMA stimulated U1 cells

PMA	NAm	Intracellular [NAD]
none	none	340 pmoles
50 ng/ml	none	300 pmoles
	5 mM	925 pmoles
	10 mM	960 pmoles

U1 cells in RPMI 1640 media; NAD quantitations were performed following 12 hours of PMA stimulation and values are given for 10^6 cells.

Table IV. NAm maintains elevated NAD in acutely infected primary cells

Post infection (hours)	Donor PBLs		
	control	#1	#2
	uninfected + 0 NAm	infected + 0 NAm	infected + 5 NAm
8	48 %	36 %	208 %
16	48 %	28 %	226 %
24	56 %	24 %	202 %

PBLs checked at 8 hour intervals over first day of acute HIV infection in culture. NAD was assayed by the enzyme cycling method for 10^7 cells. NAD values are given as a percentage of the 0 time control. At time 0, cultures recieved either 100ng of HIV-HXB_2 alone (#1), or both virus and 5mM supplemental NAm(#2).

A few groups have published data suggesting that HIV-1 is involved in the PARP pathway (14, 15, 16). In all cases there was indirect evidence for PARP activation; Furlini and co-workers for example demonstrated increased ADP-ribosylation in the protein pellet following HIV infection, and Yamogoe et. al. demonstrated that PARP inhibitors including NAm lead to transient post-transcriptional blockage of proteins under the control of the HIV-LTR. Alternatively, both of these findings are also consistent with cytoplasmic mono ADP ribosylation activation (17). We therefore raise the possibility that more than one NAD degradation pathway may be involved in the HIV life cycle (i.e. - PARP and a cytoplasmic mono ADP ribosylation step).

Rapid and irreversible depletion of NAD in response to massive DNA damage has been demonstrated with N-methyl-N-nitro-N-nitrosoguanidine and with UV radiation; this depletion has been dubbed a "suicide response" (18). Such NAD depletion and subsequent cellular death has been attributed in some cases to the induction of PARP. We agree with Furlini that it is plausible, given our combined data, that HIV infection could cause also cell death through NAD depletion (14).

Synthesis of NAD requires either niacin or tryptophan as its initial precursor. There is evidence for generalized tryptophan catabolism in HIV infected patients. Patient's plasma is notable for the specific depletion of tryptophan, and the accumulation of quinolinic acid. These changes are felt to be secondary to the activation of indolamine 2,3 dioxygenase by γ -interferon (19, 20, 21). The postulated benefit of this metabolic shunt is the deprivation of tryptophan from offending microbes (22), however, we propose that such a metabolic diversion may also be required for the biosynthesis of NAD in HIV infected cells.

To our knowledge, this is the first report to suggested that HIV-1 directly causes a state of intracellular pellagra, as a result of increased NAD degradation. This process can be reversed and the virus can be inhibited by NAm. Brown and collaborators have done a

number of studies examining the effect of HIV on tryptophan metabolism, and in one paper they recognized the systemic pellagroid features of AIDS (22). However, their hypothesis speculates that pellagroid symptoms occur secondary to altered tryptophan metabolism (22) not as a result of increased NAD degradation. In light of the problems associated with excessive tryptophan intake, Brown and coworkers suggestion that tryptophan be used as a supplement would not be advisable (23). The more direct approach of using niacin (either nicotinamide or nicotinic acid) in HIV infected patients is the logical extension of our studies, however there is no published information on this approach to date.

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