OLIGONUCLEOTIDES 18:329–336 (2008)

DOI: 10.1089/oli.2008.0152

Immunological Response to Peptide Nucleic Acid and its Peptide Conjugate Targeted to Transactivation Response (TAR) Region of HIV-1 RNA Genome

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Anti-human immunodeficiency virus-1 (HIV-1) polyamide (peptide) nucleic acids (PNAs) conjugated with cell-penetrating peptides (CPPs) targeted to the viral genome are potent virucidal and antiviral agents. Earlier, we have shown that the anti-HIV-1 PNA_{TAR}-penetratin conjugate is rapidly taken up by cells and is nontoxic to mice when administered at repeat doses of as high as 100 mg/kg body weight. In the present studies we demonstrate that naked PNA_{TAR} is immunologically inert as judged by the proliferation responses of splenocytes and lymph node cells from PNA_{TAR}-immunized mice challenged with the immunizing antigen. In contrast, PNA_{TAR}-penetratin conjugate is moderately immunogenic mainly due to its penetratin peptide component. Cytokine secretion profiles of the lymph node cells from the conjugate-immunized mice showed marginally elevated levels of proinflammatory cytokines, which are known to promote proliferation of T lymphocytes. Since the candidate compound, PNA_{TAR}-penetratin conjugate displays potent virucidal and antiviral activities against HIV-1, the favorable immunological response together with negligible toxicity suggest a strong therapeutic potential for this class of compounds.

Introduction

Polyamide nucleic acids (PNAs) are a new class of antisense DNA mimics which are devoid of sugar-phosphate backbone (Nelsen et al., 1991) and display higher affinity to complementary nucleic acid sequences than do normal oligo DNA or RNA. PNAs are constituted by an achiral, uncharged pseudopeptide backbone that makes them extremely stable in biological fluids, completely resistant to nucleases and proteases (Demidov et al., 1994). These unique properties of PNA offer greater therapeutic potential for use against viral and bacterial infections, metabolic disorders, and diseases including cancers (Branden et al.. 1999; Boffa et al., 2000; Sazani et al., 2002; Chaubey et al., 2005; Tan et al., 2005; Tripathi et al., 2005; 2007; Maier et al., 2006). Earlier, we have demonstrated strong anti-human immunodeficiency virus-1 (HIV-1) activity of PNA targeting the critical regions of the HIV-1 RNA genome. We showed that PNAs targeting the primer-binding site (PBS) and A-loop sequences in the U5-PBS region of the HIV-1 genome block the initiation of reverse transcription (Lee et al., 1998) by destabilizing the natural $tRNA_3^{Lys}$ primer from the viral genome and strongly inhibit HIV-1 replication (Kaushik et al., 2001; Kaushik and Pandey, 2002). Similarly PNAs targeted to the transactivation response (TAR) element of HIV-1 long-terminal repeat (LTR) efficiently block Tat-TAR interaction, inhibit Tat-mediated transactivation of HIV-1 LTR transcription (Mayhood et al., 2000) and HIV-1 production (Kaushik et al., 2002b).

Several approaches have been used to improve the biodelivery and efficacy of PNAs including their covalent conjugation to carriers such as neutral or cationic lipophilic molecules (Muratovska et al., 2001; Filipovska et al., 2004; Mehiri et al., 2008), cell-penetrating peptides (CPPs) (Kaushik et al., 2002a), and cell-specific receptor ligands or encapsulation in autologous erythrocytes (Boffa et al., 2000; Koppelhus and Nielsen, 2003; Chiarantini et al., 2005). We have shown that anti-HIV-1 PNA conjugated with neamine is not only efficiently taken up by the cells but also acquires a unique nuclease-like property that specifically cleaves the target RNA (Riguet et al., 2004; Chaubey et al., 2007). Recently, we discovered that anti-HIV-1 PNAs conjugated with CPP are potent virucidal agents which penetrate into HIV-1 virions and render them noninfectious. Besides, the PNA-CPP conjugates are nontoxic at repeat intraperitoneally administered doses of as high as 100 mg/kg body weight and nonlethal at a single dose of

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300 mg/kg body weight (Chaubey et al., 2008). These studies have established the therapeutic potential of this class of anti-HIV-1 PNA-CPP conjugates which are safe and possess potent antiviral and virucidal activities. In this communication we present our studies on the immune response to PNA and its CPP conjugates in the mouse model.

Materials and Methods

Materials

PNA_{TAR}-penetratin conjugate was synthesized by Bio-Synthesis, Inc., (Lewisville, Texas, USA). PNA_{TAR} was (H_2N-TCCCAGGCTCAGATCT-$COOH_2$) covalently linked at its N-terminus with penetratin peptide (H_2N-RQIKIWFQNRRMKWKK-$COOH_2$) via egl-linker (-O-). The conjugate had a molecular mass of 6898 with purity >90% as determined by high performance liquid chromatography. The lyophilized powder was dissolved in sterile phosphate buffered saline with occasional shaking for 10–15 minutes at 50°C till a clear solution was achieved.

Animals

Four-to-five-week-old female Balb/c mice were purchased from Taconic Farms, Inc. (New York, USA) and quarantined for 2 weeks before the start of the experiment. The weight variation of individual mice was within ±10% of the mean body weight. The mice were fed Lab-Diet certified Rodent Diet. Food and water were available *ad libitum*. The mice were housed in a room with a temperature of 70 ± 3°F, relative humidity of 31%–62%, and a 12-hour light/dark cycle. All husbandry conditions and experiments were performed according to the *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee at UMDNJ. Every measure was taken to ensure the welfare of the animals at all times during the course of this study.

Preparation of antigen sample for immunization

The samples of penetratin peptide, PNA_{TAR}-penetratin conjugate, or naked PNA_{TAR} were prepared by dissolving in phosphate buffered saline solution at a concentration of 2 mg/mL. The individual solutions were sterilized by passing through Millipore syringe filter (polyvinylidene fluoride, 0.45 μm) and then mixed with an equal volume of complete Freund's adjuvant. The final concentration of each antigen solution was 1 mg/mL.

Subcutaneous immunization of mice

Six-week-old female Balb/c mice were used in this study. We immunized each mouse with 0.2 mL of an individual antigen solution (penetratin peptide, PNA_{TAR}-penetratin conjugate or naked PNA_{TAR}) subcutaneously at two different sites (total of 400 μg/mouse).

Preparation of mouse splenocytes and lymph node cell suspension

Four weeks after immunization, the mice were euthanized and their lymph nodes and spleens were removed aseptically. The cell suspension was prepared by teasing the tissues with forceps and then triturating by flushing several times with a Pasteur pipette. Following centrifugation of the cell suspension at 1000 rpm for 10 minutes at 25°C, the erythrocytes were lysed by a hypotonic solution and the cells were washed twice with RPMI-1640. The cells were then resuspended in RPMI medium supplemented with 10% fetal bovine serum and 50 μM 2-mercaptoethanol (complete RPMI medium), and their number was adjusted to 10^6 cells/mL. The viability of the splenocytes and the lymph node cells was determined by the trypan-blue dye exclusion technique.

Thymidine incorporation assay

The splenocyte and lymph node cell suspensions (0.2×10^6 cell/mL) from immunized mice were cocultured with γ-irradiated (3000 R) antigen-presenting cells (0.4×10^6) in a 96-well plate in complete RPMI medium. The individual cell cultures were challenged with different concentrations of penetratin peptide, PNA_{TAR}-penetratin conjugate or naked PNA_{TAR} for 72 hours at 37°C in 5% CO_2 and then pulsed with 50 μCi of ^{3}H-thymidine for 8 hours. As positive controls, the cell cultures were also stimulated with Concanavalin A and pulsed with ^{3}H-thymidine. The cells were then harvested onto glass-fiber filters and counted for radioactivity in a Liquid Scintillation Counter (Packard).

Cytokine assays

Six-week-old female Balb/c mice were immunized subcutaneously at two different sites with 400 μg of individual antigen samples prepared as above. Four weeks after immunization, the mice were euthanized and their lymph nodes were harvested. The single-cell suspension prepared from lymph nodes was cocultured as above with γ-irradiated antigen-presenting cells in a 96-well plate and then challenged for 24 hours with either 50 μg /mL of PNA_{TAR}-penetratin or penetratin alone. The culture supernatants collected after 24 hours were analyzed for interleukin-2 (IL-2), IL-4, IL-10, IL-12, IFN-γ, and tumor necrosis factor-α (TNF-α). Concanavalin A–stimulated cells were used as the positive controls. Cytokine profiling in the presence of individual challenging antigen was performed by sandwich enzyme-linked immunosorbent assay at Cytokine Core Lab in University of Maryland, Baltimore using Biotin-streptavidin-peroxidase system. The individual cytokine concentration in the samples was calculated from their standard curves using SoftMaxPro (Molecular Devices, Sunnyvale, CA, USA).

Results

Proliferation responses of splenocytes and lymph node cells following immunization

In the first set of experiments we immunized individual mice with naked PNA_{TAR}, penetratin or PNA_{TAR}-penetratin conjugate, and then examined the proliferation responses of splenocytes and lymph node cells from each immunized mouse by challenging with different concentrations of the same immunizing antigen. The immunization of mice with

antigen is generally carried out to prime the B and T cell that leads to the formation of memory cells. These memory cells elicit rapid immune response when encountered with the same antigen. The results are shown in Figure 1. As shown in the figure, the proliferation responses of splenocytes and lymph node cells from mice immunized with naked PNA_{TAR} were the lowest (Fig. 1A), when compared with the responses obtained from the mice immunized with penetratin or PNA-penetratin conjugate (Fig. 1B and C). The proliferation responses were more or less similar in both the penetratin or PNA_{TAR}-penetratin immunized mice. We also determined the proliferation response of splenocytes from nonimmunized mice challenged with penetratin or PNA_{TAR}-penetratin conjugate (Fig. 1D). The extent of ^{3}H-thymidine incorporation in splenocytes from nonimmunized mice challenged with the conjugate (25 μg/mL) was ~80% with respect to that obtained with immunized mice (Fig. 1C). However, when challenged with penetratin, the proliferation response in nonimmunized mice (Fig. 1D) was either negligible or insignificant as compared to the response obtained with penetratin-immunized mice (Fig. 1B).

Since the proliferation responses of splenocytes and lymph node cells from mice immunized by naked PNA_{TAR} were insignificant when challenged with the same antigen (Fig. 1A), we excluded naked PNA from our next immunization experiments. In the second set of experiments we immunized mice with either penetratin (Fig. 2A) or PNA_{TAR}-penetratin conjugate (Fig. 2B) and determined the proliferation responses in the splenocytes and lymph node cells by challenging them with either the conjugate or its individual components (PNA_{TAR}, penetratin). As expected, proliferation responses in both splenocytes and lymph node cells from either conjugate or penetratin-immunized mice were negligible when challenged with naked PNA_{TAR}. In contrast, the extent of ^{3}H-thymidine incorporation in both splenocytes and lymph node cells was significantly higher when

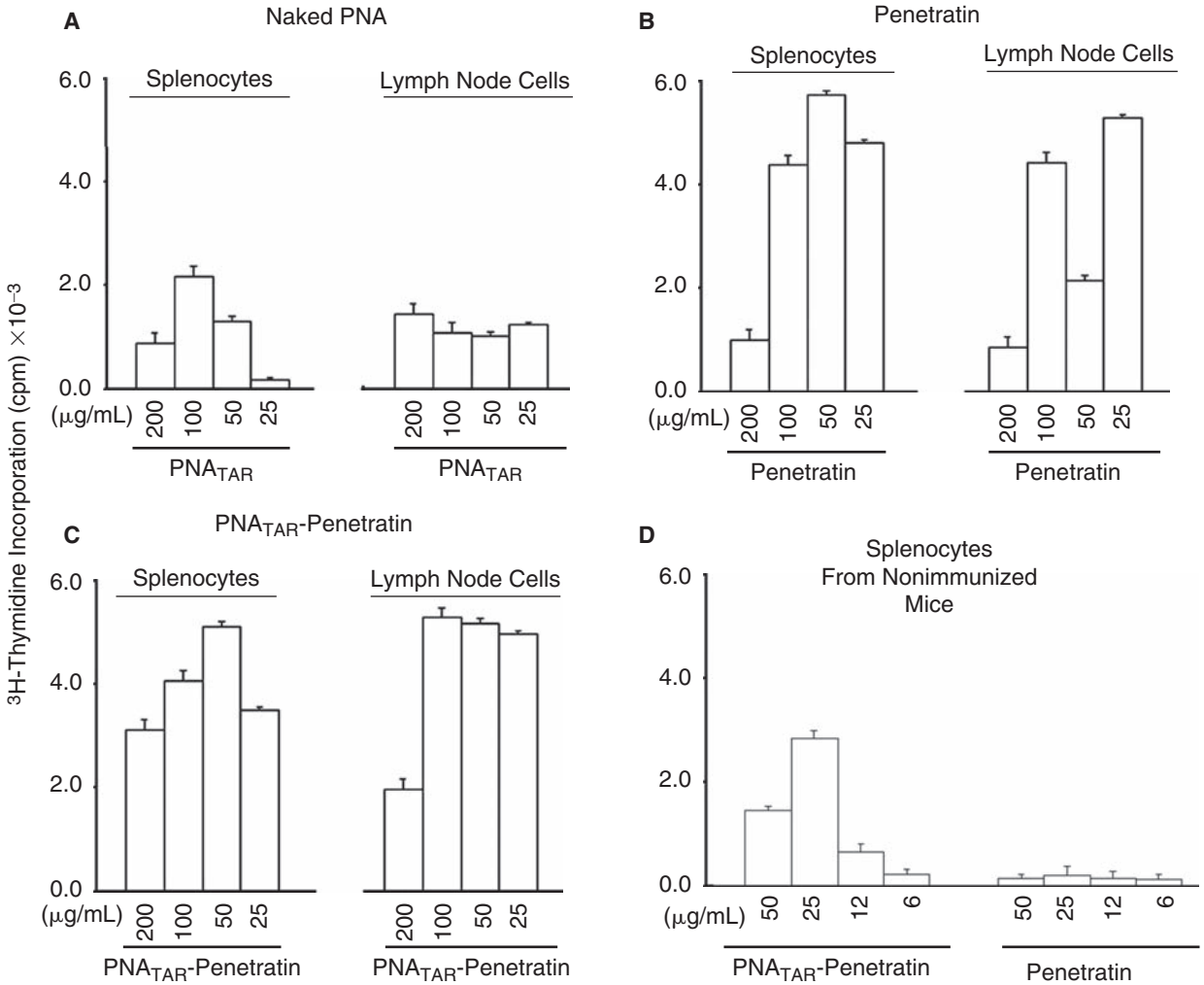

FIG. 1. Proliferation responses of splenocytes and lymph node cells from immunized mice challenged with the same immunizing antigen. The splenocytes and lymph node cells from mice immunized by either naked PNA_{TAR} (**A**), penetratin (**B**), PNA_{TAR}-penetratin conjugate (**C**), or from nonimmunized mice (**D**) were challenged with individual immunizing antigens and pulsed with ^{3}H-thymidine as described in the Materials and Methods. The cells were harvested and counted for radioactivity. The results depicted are an average of three sets of experiments.

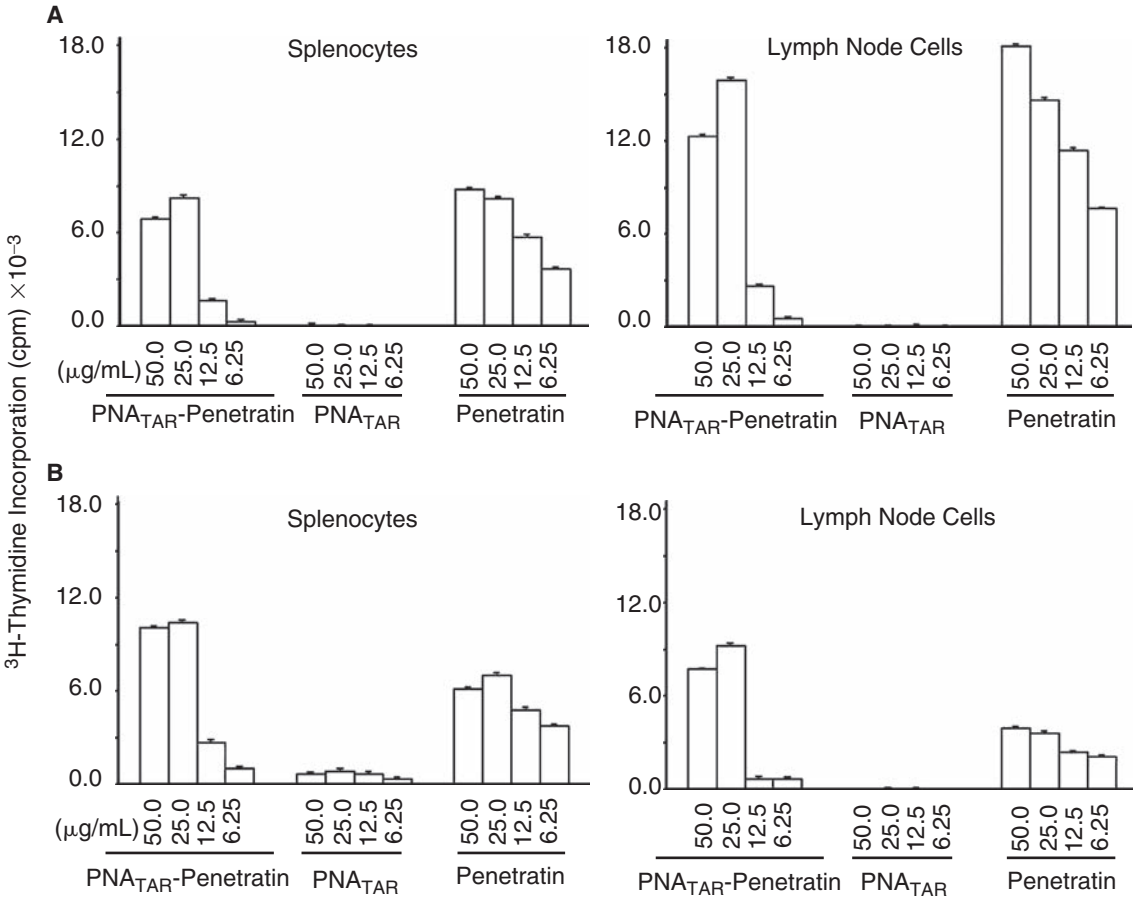

FIG. 2. Proliferation responses of splenocytes and lymph node cells from penetratin-immunized (**A**) or conjugate-immunized (**B**) mice challenged with naked PNA, penetratin, and the conjugate. Splenocytes and lymph node cells from immunized mice were grown in triplicate in a 96-well plate and challenged with individual antigens at indicated concentrations and pulsed with labeled thymidine as described in the Materials and Methods. The cells were harvested and counted for incorporated radioactivity. The results shown are averages of three sets of experiments.

challenged with either penetratin or PNA$_{TAR}$-penetratin conjugate. These results indicated that PNA alone is nearly non-immunogenic while PNA-penetratin conjugate or its peptide component (penetratin) is moderately immunogenic.

Cytokine secretion by lymph node cells following immunization with PNA$_{TAR}$-penetratin conjugate or penetratin peptide

Cytokine secretion by penetratin or PNA$_{TAR}$-penetratin stimulated lymph node cells from mice immunized by the respective antigen was compared (Table 1). Con A stimulated lymph node cells from mice immunized by either penetratin or PNA$_{TAR}$-penetratin conjugate was used as positive controls and their cytokine secretion was determined. In a parallel set of experiments, we also determined the proliferation response of lymph node cells challenged with the polyclonal T-cell mitogen, Con A, PNA$_{TAR}$-penetratin, or penetratin alone (Fig. 3). The lymph node cells were stimulated for 24 hours and the culture supernatants were assayed for IL-2, IL-4, IL-10, IL-12, IFN-γ, and TNF-α. As shown in the table, lymph node cells from mice immunized with PNA$_{TAR}$-penetratin conjugate when challenged with the same antigen secreted significant levels of IL-2, IL-12, IFN-γ, and TNF-α while the amounts of IL-4 and IL-10 were below detectable levels for the assay. Similar results were obtained with penetratin-immunized mice challenged with penetratin, although IL-2 secretion level was 30% lower than that obtained with conjugate immunized mice. However, the levels of IL-12 and IFN-γ produced by penetratin stimulated lymph node cells were 2- and 8-fold higher, respectively, than those of conjugate stimulated cells.

Discussion

This is the first study evaluating the immune response to PNA and PNA-penetratin conjugate in mice. Although penetratin peptide has been shown to suppress the inflammatory response by inhibiting activation and nuclear translocation of nuclear factor-κB (Letoha et al., 2006) in rats, its specific immunogenic response is not known. Recently Moschos et al. (2007) have used penetratin to deliver small-interfering RNA (siRNA) in lung and shown that siRNA-penetratin conjugate bring forth innate immune response by inducing TNF-α, IFN-α and IL-12 in mice. In another report, a CpG DNA oligo has been demonstrated to be highly proinflammatory by triggering TNF-α response in both cultured macrophages and in mice while PNA oligo with similar CG sequences

Table 1. Cytokine Secretion Profile of Lymph Node Cells from Muse Immunized by Either Penetratin or PNA_{TAR}-Penetratin Conjugate

Cytokines	*Lymph node cells from mice immunized by*			
	Penetratin and challenged with:		*PNA_{TAR}-penetratin and challenged with:*	
	Penetratin	*Con A*	*PNA_{TAR}-penetratin*	*Con A*
	Cytokine produced (pg/mL)			
IL-2	21.4 ± 1.9	1670 ± 169	29.3 ± 3.5	1980 ± 321
IL-4	Low	14.6 ± 0.8	Low	27.9 ± 0.4
IL-10	Low	93 ± 1.9	Low	141 ± 15
IL-12	10.1 ± 1.5	13.0 ± 0.1	5.8 ± 2.0	20.9 ± 6.8
IFN-γ	17.1 ± 2.8	624 ± 3.5	2.03 ± 0.2	567 ± 55
TNF-α	8.44 ± 1.4	155 ± 8.4	6.91 ± 0.74	168 ± 22

Balb/c mice were immunized subcutaneously by either penetratin or the conjugate. Four weeks after immunization, the lymph nodes were harvested and a single-cell suspension was grown and challenged by the immunizing antigen and also by Con A as described in the Materials and Methods. After 24 hours, the culture supernatant of each set was collected and analyzed for indicated cytokines by enzyme-linked immunosorbent assay in triplicate.

IL, interleukin; TNF, tumor necrosis factor.

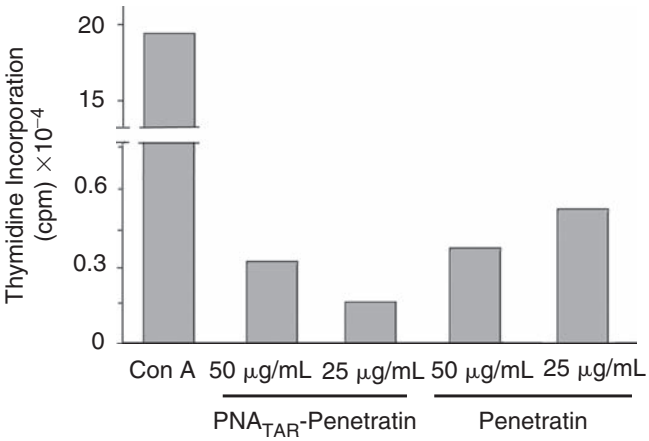

FIG. 3. Proliferation response of lymph node cells from conjugate immunized mice challenged with Con A, conjugate, and penetratin. Lymph node cells from mice immunized by PNA_{TAR}-penetratin conjugate were grown in triplicate and challenged with 10 μg/mL Con A or indicated concentrations of the conjugate or penetratin as described in the Materials and Methods. The cells were pulsed with ^3H-labeled thymidine for 8 hours and the extent of thymidine incorporation was determined as described in the Materials and Methods. The results shown are averages of three sets of experiments.

was found to be immunologically inert (Yuan et al., 2003). Another PNA oligo (PNAEμ) that blocks c-myc hyper expression in Burkitt's lymphoma failed to illicit antigenic immune response in mice while the same PNAEμ crosslinked to the immunogenic carrier keyhole limpet hemocyanin was antigenic (Cutrona et al., 2007). In the present studies, we also noted that the immune response to naked PNA_{TAR} was the lowest when compared with the immune response to PNATAR-penetratin conjugate or penetratin alone. We determined the proliferation responses of splenocytes and lymph node cells from mice immunized with individual antigens by challenging with the same immunizing antigen. The responses obtained with naked PNA_{TAR} as immunizing and challenging antigen were negligible and were the lowest in comparison with the responses to penetratin and PNA_{TAR}-penetratin conjugate (Fig. 1). Our results together with the previous studies (Yuana et al., 2003; Cutrona et al., 2007) suggest that irrespective of the PNA sequences, naked PNAs are immunologically inert molecules. The proliferation responses, which we obtained with PNA_{TAR}-penetratin conjugate, could be due to penetratin component of the conjugate. This premise was supported when splenocytes and lymph node cells from mice immunized by penetratin or PNA_{TAR}-penetratin conjugate were challenged individually with conjugate or its constituents, naked PNA_{TAR}, and penetratin (Fig. 2). The proliferation responses were significant when challenged with either penetratin or PNA-penetratin conjugate but not when challenged with naked PNA.

Further analysis of the cytokine secretion profile of lymph node cells from mice immunized by either PNA-penetratin conjugate or penetratin and then challenged by the immunizing antigen yielded very interesting results. We noted that the secretion of IL-2 by lymph node cells was the highest when challenged with either penetratin or PNA-penetratin conjugate (Table 1). Since the candidate compound, PNA_{TAR}-penetratin conjugate, is a potent virucidal agent against HIV-1 and displays strong HIV-1 antiviral activity, the immunological response elicited by the conjugate with respect to the elevated production of IL-2 is expected to be beneficial to the patient. Although IL-2 exerts its effects on many cell types, the most prominent effect of this cytokine is on the activation of T lymphocytes (Kremer et al., 1996; Snijders et al., 1998; Lenardo et al., 1999, Wang et al., 2000). It promotes proliferation of T lymphocytes and increases the count of mature T cells and also slows down the death of CD4+ T cells. HIV-1 infection not only decreases the CD4+ cell count and endogenous levels of IL-2 but also causes an increase in the dysfunction of existing CD4+ cells. These three deleterious effects of HIV-1 infection are potentially reversible with the administration of recombinant IL-2 (Conard, 2003). A number

of clinical trials on HIV-1 patients undergoing antiretroviral therapy with IL-2 supplement have shown a significant increase in the number of their CD4+ cells, and decrease in HIV/AIDS-related morbidity as compared to patients receiving the same therapy without IL-2 supplement (Kovacs et al., 1996; Arnó et al., 1999; Levy et al., 1999; Davey et al., 2000; Emery et al., 2000; Losso et al., 2000; Ruxrungtham et al., 2000; Abrams et al., 2002).

Besides IL-2, IL-12, interferon-γ (IFN-γ), and TNF-α were also produced by the lymph node cells from immunized mice. IL-12 stimulates the growth and function of T cells and the production of IFN-γ and TNF-α. Both IL-12 and IL-2 are important cytokines in the regulation of a cell-mediated immune response; IL-2 being responsible for stimulating the proliferation of T cells, while IL-12 promotes the differentiation of the antigen-activated CD4+ T cells into TH1 cell type (Hsieh et al., 1993). The production of proinflammatory cytokines (IL-2, IFN-γ, TNF-α), but not anti-inflammatory cytokines (IL-4, IL-10) from lymph node cells stimulated with penetratin and PNA_{TAR}-penetratin conjugate is consistent with TH1 cell differentiation, which supports cell-mediated immune responses (e.g., cytotoxic T lymphocytes, natural killer cells, activated macrophages) that would benefit patients with HIV-1 infection.

In conclusion, our results suggest that while naked anti-HIV-1 PNA_{TAR} is an immunologically inert molecule, its penetratin peptide conjugate is moderately antigenic. However, the immune responses elicited by PNA_{TAR}-penetratin conjugate are beneficial to the host since it induces elevated levels of type 1 cytokines such as IL-2 and IL-12 which are known to stimulate T-cell proliferation and slow down the death of CD4+ T cells. We have earlier shown that anti-HIV-1 PNA-CPP conjugates targeting critical regions of HIV-1 RNA genome are efficiently taken up by cells and display both antiviral and virucidal activities against HIV-1 (Kaushik et al., 2002a; Chaubey et al., 2005; Tripathi et al., 2005; 2007). We have recently shown that PNA-CPP conjugates are nontoxic to mice up to a single 300-mg dose/kg of body weight and well tolerated when administered in repeat doses of 100 mg/kg of body weight (Chaubey et al, 2008). The low or negligible *in vivo* toxicity and stability of PNA and its CPP conjugates, as well as their slow release and clearance from different organs (Ganguly et al., 2008) together with the beneficial immune responses that they induce suggest the possible therapeutic potential of this class of compounds.

Acknowledgment

This research was supported by a grant from the NIH/NIAID (AI042520) to V.N.P.

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Received for publication July 16, 2008; accepted after revision September 27, 2008.