Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 63 (2004) 133–138
Antiviral Research
www.elsevier.com/locate/antiviral

Short communication

Tenofovir primes rhesus macaque cells in vitro for enhanced interleukin-12 secretion

Koen K.A. Van Rompay [a,*], Marta L. Marthas [a], Norbert Bischofberger [b]

[a] *California National Primate Research Center, University of California, Davis, CA 95616, USA*
[b] *Gilead Sciences, Foster City, CA, USA*

Received 23 February 2004; accepted 9 April 2004

Abstract

We investigated if the antiviral drug tenofovir has immunomodulatory effects in macaques, similar to those described in murine models. While in vivo experiments were complicated by high individual and temporal variability of immune parameters, tenofovir primed macaque peripheral blood mononuclear cells in vitro for enhanced IL-12 secretion following exposure to bacterial antigens.

Keywords: Tenofovir; In vitro; IL-12; Macaque; PMPA

1. Introduction

In murine systems, immunomodulatory effects have been demonstrated extensively for the antiviral nucleotide analogues tenofovir (9-[2-(phosphonomethoxy)propyl]adenine; PMPA) and adefovir (9-(2-phosphonomethoxyethyl)adenine; PMEA). In vitro studies with murine cells found that tenofovir by itself did not affect nitric oxide production, but increased the production of nitric oxide induced by exogenous interferon-gamma (IFN-γ) through a post-translational mechanism (Zídek et al., 2000, 2001, 2003). Tenofovir also stimulated secretion of tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10), IL-1β, macrophage inflammatory protein 1-alpha (MIP1-α) and "regulated-upon-activation, normal T cell expressed and secreted" (RANTES) by peritoneal macrophages or splenocytes, but did not affect the production of other cytokines, such as IL-2, IL-12, and IFN-γ (Zídek et al., 2001, 2003). Consistent with these data, in vivo treatment of mice with tenofovir resulted in increased serum levels of TNF-α, IL-10, and RANTES (Zídek et al., 2001). In contrast, adefovir inhibited RANTES secretion and had no effect on nitric oxide production in vitro (Zídek et al., 1999, 2001, 2003). Adefovir has extensive in vivo immunomodulatory effects in murine models, including anti-tumor and anti-arthritis activity and increased IFN-α/β production (Caliò et al., 1994; Del Gobbo et al., 1991; Otová et al., 1993a,b,c, 1997; Villani et al., 1994; Zídek et al., 1995). Experiments in mice infected with influenza A virus have shown that adefovir is partially protective in reducing the mortality caused by this RNA virus, which is insensitive to the direct antiviral effect of adefovir in vitro (Villani et al., 1994). Both tenofovir and adefovir have been found to enhance NK cell activity in mice (Caliò et al., 1994; M. Ussery, personal communication).

Simian immunodeficiency virus (SIV) infection of macaques is a valuable animal model of HIV infection and AIDS. In comparison to other antiviral drugs, tenofovir has been highly effective in this animal model to prevent infection, or treat established infection (Van Rompay et al., 1996, 2004b). Several unexpected observations in SIV-infected macaques suggest that tenofovir may have immuno-preserving or -stimulatory effects in vivo. First, even after the emergence of drug-resistant viral mutants, animals can maintain low or undetectable viremia for years due to the development of effective antiviral CD8+ cell-mediated immune responses (Van Rompay et al., 2004b). In addition, tenofovir treatment prolonged disease-free survival of SIV-infected macaques even in the presence of high viremia, suggesting improved defense mechanisms against opportunistic infections (Van Rompay et al., 1999, in press). To investigate whether direct immunomodulatory effects (i.e., independent of antiviral activity) of tenofovir may contribute to these observations in SIV-infected macaques, several in vitro and in vivo

* Corresponding author. Tel.: +1-530-752-5281; fax: +1-530-754-4411.
E-mail address: kkvanrompay@ucdavis.edu (K.K.A. Van Rompay).

0166-3542/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2004.04.002

experiments, mimicking the murine experiments, were performed with rhesus macaques. All animals were SIV-free, type D-retrovirus-free and healthy, and were housed in accordance with American Association for Accreditation of Laboratory Animal Care standards; we adhered to the "Guide for Care and Use of Laboratory Animals" (National Research Council, 1996).

2. In vitro studies with rhesus macaque cells

2.1. Effect of tenofovir on NK cell activity

Heparin-anti-coagulated blood was collected from healthy rhesus macaques. Peripheral blood mononuclear cells (PBMC) were isolated using standard ficoll-gradient separation techniques, washed and incubated in RPMI-1640 medium (Irvine Scientific, Santa Ana, CA), supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products Inc., Calabasas, CA), penicillin/streptomycin and L-glutamine (Gibco BRL, Grand Island, NY). In some experiments, rhesus macaque monocyte-derived macrophage cultures were obtained and maintained as described previously (Miller et al., 1998). In a first set of experiments, PBMC were incubated overnight in the presence of different concentrations of tenofovir (0–100 μM) followed by a standard 4-h NK cell assay (^{51}Cr release assay with K562 cells). In a second set of experiments, monocyte-derived macrophages incubated with tenofovir overnight or their culture supernatants were added to freshly isolated PBMC, and the next day ^{51}Cr-labeled K562 cells were added for a standard NK cell assay. In both sets of experiments, no significant effect of tenofovir on NK cell activity was observed, while the addition of IL-2 (50 U/ml) as a positive control increased NK cell activity significantly (data not shown).

2.2. Effect of tenofovir on nitric oxide production

We investigated if tenofovir affects nitric oxide production similarly to what has been described in murine macrophages (Zídek et al., 1997). No nitric oxide could be detected (i.e., <0.4 μM, measured using the Griess reagent; Zídek et al., 1997) in rhesus macaque monocyte-derived macrophage cultures following in vitro stimulation with human IFN-γ (100 or 1000 U/ml; R&D, Minneapolis, MN), lipopolysaccharide (LPS; 1 μg/ml; from *Escherichia coli*; Sigma), or both, for incubation times up to 6 days. The addition of tenofovir to the macrophage cultures did also not result in detectable nitric oxide levels. This difficulty to detect nitric oxide in macaque macrophage cultures is consistent with observations for human macrophage cultures (Albina, 1995; Denis, 1994), indicating that nonhuman primates are closer models for human immunology than murine systems.

2.3. Effect of tenofovir on cytokine secretion

Tenofovir (in concentrations ranging from 0 to 200 μM) had no significant effect on RANTES secretion in rhesus macaque PBMC (measured by the Quantikine® human RANTES ELISA kit, R&D Systems, Minneapolis, MN; data not shown). In another set of experiments, rhesus macaque PBMC or monocyte-derived macrophage cultures were incubated with tenofovir (ranging from 0 to 500 μM) and supernatants were collected 2 days later and tested for the presence of IL-2, IL-4, IL-10, IL-12p40, IFN-γ, and TNF-α by commercially available rhesus macaque-specific ELISA kits (Biosource International, Camarillo, California; U-CyTech, Utrecht, the Netherlands). Tenofovir did not induce detectable secretion of any of these cytokines. In the next set of experiments, rhesus macaque PBMC were incubated with tenofovir overnight, and then stimulated with SAC (Staphylococcus aureus Cowan strain; 0.0075% w/v; Calbiochem, San Diego, CA), a known inducer of IL-12. Aliquots of supernatant were collected either at 24 h or at multiple time points after the addition of SAC, frozen at −80 °C and subsequently tested by the rhesus macaque IL-12 specific ELISA (which measures both p40 and the p70 heterodimer). The kinetics and peak of IL-12 production varied highly among individual donor animals, and the often rapid decline after the initial peak suggests uptake of IL-12 by the cells. Similar to observations for human cells, SAC-induced IL-12 production in rhesus macaque PBMC was increased after incubation with human IFN-γ (10 U/ml; data not shown). Our experiments found that tenofovir induced a statistically significant, dose-dependent increase in IL-12 secretion especially very early (2 h) and late (8–24 h) after the addition of SAC, while at intermediate time points (such as 4 h), IL-12 levels could be in reverse order (Fig. 1A and B). To investigate if tenofovir also primed IL-12 production to bacterial antigens which induce IL-12 secretion through different cell receptors (Ozinsky et al., 2000; Trinchieri, 1998), similar studies were performed with LPS (1 μg/ml). While IL-12 secretion following exposure to LPS was slower and of lower magnitude than that following SAC, similar priming effects of tenofovir were observed (Fig. 1C and D). Although IFN-γ was usually undetectable in supernatants collected 24 h after SAC or LPS stimulation (i.e., it may require longer incubation), the limited available data where IFN-γ levels were detectable suggest that the tenofovir-induced increased IL-12 secretion following SAC stimulation was associated with increased IFN-γ levels (data not shown).

A similar in vitro stimulation experiment was also performed with rhesus macaque PBMC to measure cytokine and chemokine mRNA levels. Freshly isolated PBMC were incubated in 6-well plates in the absence or presence of tenofovir (50 μM), and some cultures received SAC (0.0075% w/v) 4 h later. After different times of incubation, cells were harvested (including treatment of the wells with 0.02% EDTA/PBS (Sigma) to detach adherent cells), washed, dissolved in Trizol reagent (Gibco BRL) and stored at −80 °C.

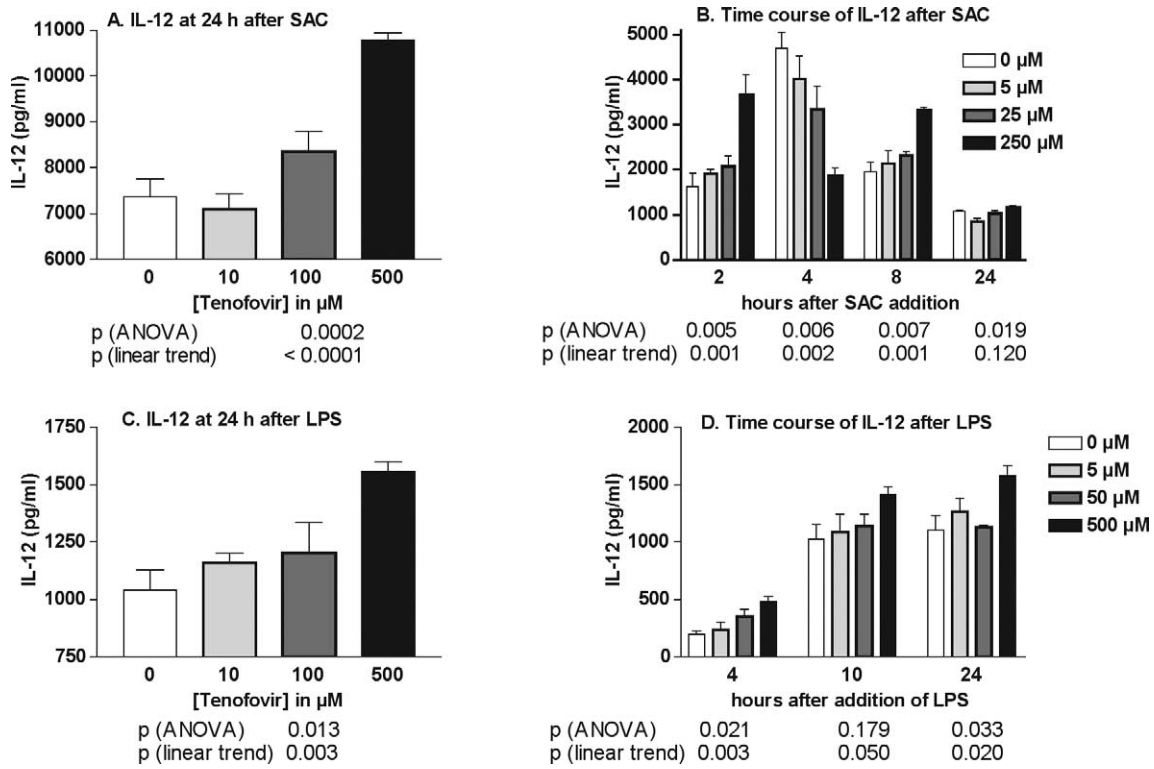

Fig. 1. In vitro immunomodulatory effects of tenofovir on IL-12 secretion. PBMC from SIV-negative rhesus macaques were incubated with tenofovir overnight, and then stimulated with SAC or LPS. IL-12 levels in supernatant collected at different times after the addition of the bacterial antigen were measured using rhesus macaque-specific ELISA kits. While there are substantial differences in the timing and magnitude of peak IL-12 secretion by PBMC among individual animals, the enclosed graphs are representative data derived from independent experiments with three SIV-negative animals. Bars represent the mean ± S.E.M. of triplicate cultures of one animal per graph. Within each graph, different color intensities represent different concentrations of tenofovir (in μM) for a constant concentration of SAC or LPS. The concentration of SAC in the experiments of graphs in panels A and B was 0.0075% (w/v); for graphs in panels C and D, the concentration of LPS was 1 μg/ml. *P* values for ANOVA and linear trend (for tenofovir concentration) were calculated using Instat 3 (GraphPad Software Inc., San Diego, CA).

These samples were subsequently used for quantitation of cytokine and chemokine mRNA by real-time PCR, according to methods described previously (Abel et al., 2001). The cytokine/chemokine panel that was tested consisted of TNF-α, MIP1-α, MIP1-β, IFN-α, IFN-β, Mx, oligoadenylate synthetase (OAS), interferon-gamma-inducible protein 10 (IP-10), monokine-induced by IFN-γ (MIG), IL-2, IL-4, IL-6, IL-10, and IL-12. In vitro incubation of PBMC (in medium containing 10% fetal bovine serum, but without tenofovir or SAC) for periods of 4–28 h already induced >10-fold increased expression of most cytokine and chemokine mRNA's (with exception of IFN-γ and IL-10, which increased approximately two- to four-fold, and IFN-α, IL-2 and IL-4, which remained similar or decreased slightly; Fig. 2). This increased mRNA expression is presumably due to stimulation of cells by the plastic surface of the tissue culture plates, or by the fetal bovine serum in the tissue culture medium. Based on experience with in vitro cytokine mRNA experiments, increases in cytokine mRNA levels in PBMC cultures were considered to be relevant only if the levels were at least two-fold higher than the baseline level at time zero (i.e., prior to the addition of tenofovir) and also at least two-fold higher than the levels in the untreated control cultures at the corresponding time point (to control for spontaneous cytokine mRNA induction during the incubation process). In comparison to placebo-treated PBMC cultures, tenofovir-exposed cultures did not have increased mRNA levels of any of these cytokines and chemokines at any time point. While stimulation with SAC induced expression of most cytokine and chemokine mRNA's, although with different temporal patterns (>10-fold increases in peak mRNA for TNF-α, IFN-γ, IFN-α, and IL-10; see Fig. 2), tenofovir did not further increase these cytokine mRNA levels. Although the positive effect of tenofovir on IL-12 secretion at the protein level (measured by ELISA) was not confirmed at the mRNA cytokine level, this is possible because of the complex regulation of IL-12 transcription, post-translational processing and secretion (reviewed by Trinchieri, 2003). As mentioned above, tenofovir increased IFN-γ-induced nitric oxide production in murine cells also not at the level of transcription but through a post-translational mechanism (Zídek et al., 2000, 2001, 2003). Accordingly, further research is needed to elucidate these mechanisms.

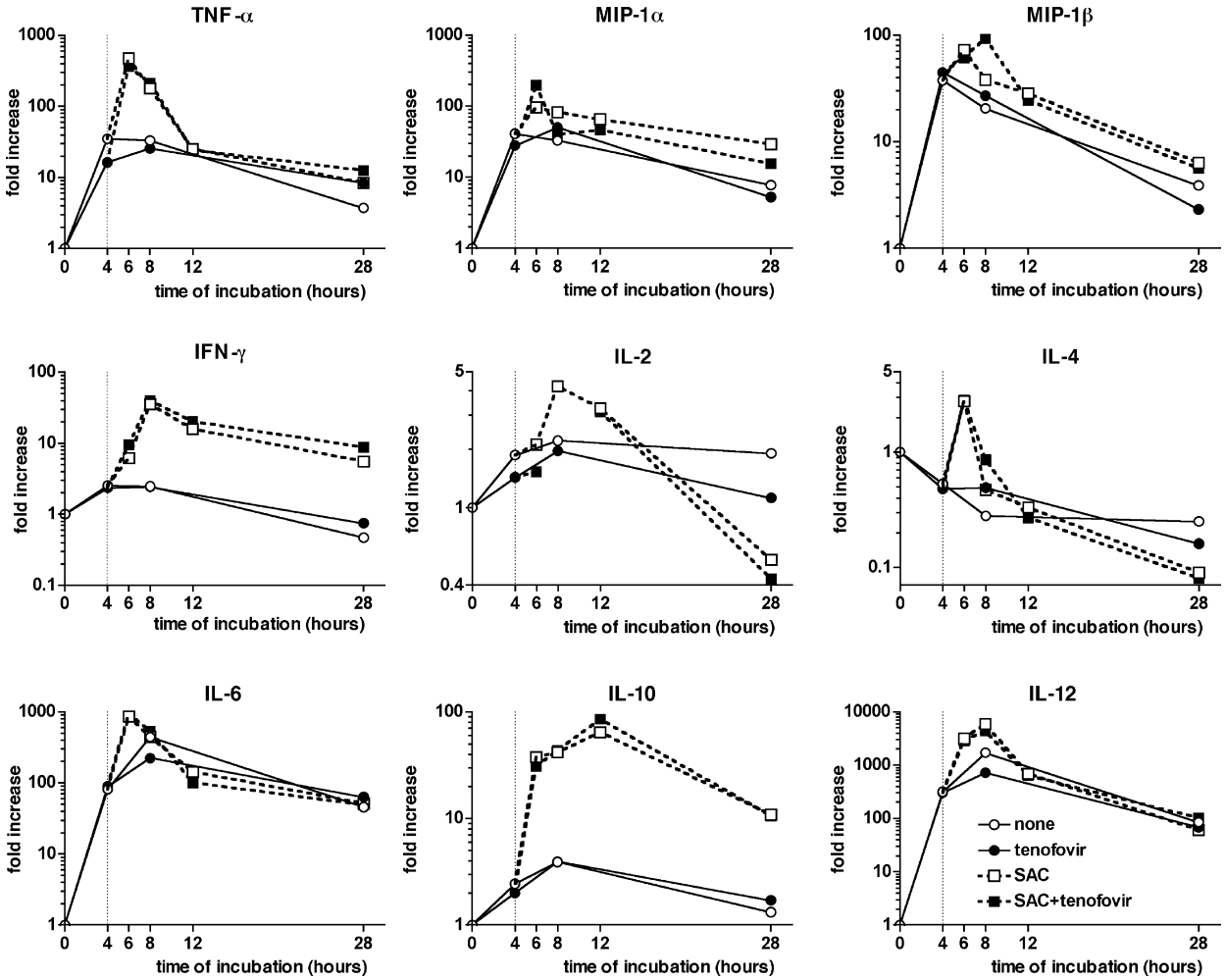

Fig. 2. In vitro effects of tenofovir on cytokine and chemokine mRNA expression in rhesus macaque PBMC. Freshly isolated PBMC of a rhesus macaque were added to wells of 6-well tissue culture plates (time zero) and immediately thereupon tenofovir (50 μM) or placebo (phosphate-buffered saline) was added. Some cultures received SAC (0.0075% w/v) 4 h later (dotted line). Cells were harvested at different times (one sample per experimental condition, and per time point). Levels of cytokine and chemokine mRNA were measured by real-time PCR techniques (using duplicate testing of each RNA sample, with standard errors less than 5%) and are expressed relative to those of the sample collected at time zero. The same legend as indicated for the IL-12 graph applies to the other graphs. Tenofovir had no significant effect on mRNA levels, including those of IFN-α, IP-10, Mx, OAS and MIG (data not shown).

3. In vivo studies in macaques

We evaluated whether tenofovir treatment induces increased NK cell activity and plasma IL-12 levels in primates. To control as much as possible for potential stress-induced immunologic changes associated with drug administration, seven SIV-seronegative juvenile rhesus macaques were started on daily subcutaneous placebo injections (sterile 0.9% NaCl). Two weeks later, for five of these seven animals, the placebo injection was replaced by tenofovir (10 mg/kg, once daily subcutaneously) for 2 weeks, followed by 4 weeks of placebo, then another 3-week treatment course of tenofovir (30 mg/kg once daily subcutaneously), followed by another 4 weeks of placebo. The other two animals received daily placebo injections throughout the whole experiment. Heparin-anti-coagulated blood samples were collected weekly, each time between 8.00 and 9.00 a.m. (to minimize the effect of any potential diurnal patterns). Standard flow cytometry techniques were used to measure lymphocyte counts, including NK cells (defined as CD3−CD8+ lymphocytes), CD8+CD3+ T lymphocytes, CD4+CD3+ T lymphocytes, and CD20+CD3− B lymphocytes (Van Rompay et al., 2003). NK cell activity was measured on freshly isolated PBMC using the standard ^{51}Cr release assay with K562 cells. Plasma was tested by the rhesus macaque-specific cytokine ELISA's described above. While plasma levels of TNF-α, IFN-γ, IL-2, IL-4, and IL-10 were all below the limit of detection throughout the whole study period, IL-12 could be detected in all plasma samples. During tenofovir treatment, no significant changes were observed for IL-12 levels in plasma, counts of lymphocytes and their subsets (including NK cells), or NK cell activity, but the considerable temporal and individual variation that was observed for each of these parameters even

among the placebo-treated control animals (of an outbred, stress-prone species) would not have allowed the detection of subtle changes (data not shown); similar variability has previously been reported for other immune markers of macaques (Boccia et al., 1997; Capitanio et al., 1998). In addition, immunological events that occur in the lymphoid tissues and mucosal sites may not be detected in peripheral blood samples (Abel et al., 2001). Further, the in vitro data (see above) suggested that tenofovir alone may not increase IL-12 production, but may prime for more rapid IL-12 production following exposure to microbial antigens; this effect of a secondary bacterial stimulus, which is difficult to control in macaques which are exposed to microbes in their regular housing environment, was not addressed in these in vivo studies.

4. Discussion

The pilot experiments described here highlight the differences in immunology between mice and primates, and the difficulty of extrapolating immunomodulatory effects from murine models to primate models of human disease (Mestas and Hughes, 2004). Preliminary in vitro observations demonstrate that tenofovir primed cells for enhanced IL-12 secretion following a secondary stimulus. Although these in vitro effects were most evident at relatively high concentrations of tenofovir ($\geq$100 μM), the tenofovir regimen used in most macaque studies (30 mg/kg once daily subcutaneously) gives peak plasma levels of $\sim$100–200 μM (Van Rompay et al., 2004a). While this in vitro priming effect on IL-12 was still relatively small (Fig. 1), IL-12 is a very potent molecule with many biological effects on innate and acquired immune responses (i.e., activation of NK cells and CTL's, stimulation of interferon-γ production and Th1 immune responses, anti-apoptotic effects; for review, see Trinchieri, 1998). Thus, an even relatively small increase of IL-12 secretion during the initial stages of an emerging immune response against a viral infection that induces immunosuppression can shift the balance towards an advantage for the host, and this can be amplified in subsequent steps. In infected individuals, such potential immunomodulatory effects of tenofovir are likely to be complemented by its antiviral effects, especially because tenofovir is very effective in vitro in inhibiting virus replication in antigen-presenting cells, which are important producers of IL-12 and play an important role in SIV and HIV disease pathogenesis (Aquaro et al., 2002; Balzarini et al., 2002; Chougnet et al., 2002; Igarashi et al., 2001; Knight, 2001; Macatonia et al., 1992; Robbins et al., 1998). Taken together, these effects could result in immunological benefits in vivo, consistent with the clinical findings we observed for tenofovir-treated SIV-infected macaques (see Section 1).

Further research, however, is definitely needed to confirm whether such immunomodulatory events, that are independent of tenofovir's antiviral effects, also occur in vivo and contribute to the observed efficacy of tenofovir in the SIV-macaque model, or in HIV-infected patients.

Acknowledgements

For their technical assistance, we thank N. Aguirre, D. Bennett, L. Fritts, L. Hirst, A. Spinner, W. von Morgenland, the Veterinary Staff, Colony Services, Clinical Laboratory and the Immunology Core of the California National Primate Research Center (CNPRC). We thank K. Abel (CNPRC), M. Miller (Gilead Sciences), and P. van der Meide (U-CyTech, Utrecht, the Netherlands) for excellent technical advice and useful discussions.

This work was supported by E. Glaser Pediatric AIDS Foundation Grant PG-50757 (K.V.R.), Gilead Sciences, and Public Science Health Grant RR00169 from the National Center for Research Resources.

References

Abel, K., Alegria-Hartman, M.J., Zanotto, K., McChesney, M.B., Marthas, M.L., Miller, C.J., 2001. Anatomic site and immune function correlate with relative cytokine mRNA expression levels in lymphoid tissues of normal rhesus macaques. Cytokine 16, 191–204.

Albina, J.E., 1995. On the expression of nitric oxide synthase by human macrophages. Why no NO? J. Leukoc. Biol. 58, 643–649.

Aquaro, S., Calió, R., Balzarini, J., Bellocchi, M.C., Garaci, E., Perno, C.F., 2002. Macrophages and HIV infection: therapeutical approaches toward this strategic virus reservoir. Antiviral Res. 55, 209–225.

Balzarini, J., Van Herrewege, Y., Vanham, G., 2002. Metabolic activation of nucleoside and nucleotide reverse transcriptase inhibitors in dendritic and Langerhans cells. AIDS 16, 2159–2163.

Boccia, M.L., Scanlan, J.M., Laudenslager, M.L., Berger, C.L., Hijazi, A.S., Reite, M.L., 1997. Juvenile friends, behavior, and immune responses to separation in bonnet macaque infants. Physiol. Behav. 61, 191–198.

Calió, R., Villani, N., Balestra, E., Sesa, F., Holy, A., Balzarini, J., De Clercq, E., Perno, C.F., Del Gobbo, V., 1994. Enhancement of natural killer activity and interferon induction by different acyclic nucleoside phosphonates. Antiviral Res. 23, 77–89.

Capitanio, J.P., Mendoza, S.P., Lerche, N.W., 1998. Individual differences in peripheral blood immunological and hormonal measures in adult rhesus macaques (*Macaca mulatta*): evidence for temporal and situational consistency. Am. J. Primatol. 44, 29–41.

Chougnet, C., Shearer, G.M., Landay, A.L., 2002. The role of antigen-presenting cells in HIV pathogenesis. Curr. Infect. Dis. Rep. 4, 266–271.

Del Gobbo, V., Foli, A., Balzarini, J., De Clercq, E., Balestra, E., Villani, N., Marini, S., Perno, C.F., Calio, R., 1991. Immunomodulatory activity of 9-(2-phosphonylmethoxyethyl)adenine (PMEA), a potent anti-HIV nucleotide analogue, on in vivo murine models. Antiviral Res. 16, 65–75.

Denis, M., 1994. Human monocytes/macrophages: NO or no NO? J. Leukoc. Biol. 55, 682–684.

Igarashi, T., Brown, C.R., Endo, Y., Buckler-White, A., Plishka, R., Bischofberger, N., Hirsch, V.M., Martin, M.A., 2001. Macrophages are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4+ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): implications for HIV-1 infections of humans. Proc. Natl. Acad. Sci. U.S.A. 98, 658–663.

Knight, S.C., 2001. Dendritic cells and HIV infection immunity with viral transmission versus compromised cellular immunity? Immunobiology 204, 614–621.

Macatonia, S.E., Gompels, M., Pinching, A.J., Patterson, S., Knight, S.C., 1992. Antigen presentation by macrophages but not by dendritic cells in human immunodeficiency virus (HIV) infection. Immunology 75, 576–581.

Mestas, J., Hughes, C.C.W., 2004. Of mice and not men: differences between mouse and human immunology. J. Immunol. 172, 2731–2738.

Miller, C.J., Marthas, M., Greenier, J., Lu, D., Dailey, P.J., Lu, Y., 1998. In vivo replication capacity rather than in vitro macrophage tropism predicts efficiency of vaginal transmission of simian immunodeficiency virus or simian/human immunodeficiency virus in rhesus macaques. J. Virol. 72, 3248–3258.

Otová, B., Sladká, M., Votruba, I., Holy, A., Kren, V., 1993a. Cytostatic effect of 9-(2-phosphonomethoxyethyl)adenine (PMEA): I. Lymphatic leukemia KHP-Lw-I in Lewis rats. Folia Biol. 39, 136–141.

Otová, B., Krenová, D., Zidek, Z., Holy, A., Votruba, I., Kren, V., 1993b. Cytostatic effect of 9-(2-phosphonomethoxyethyl)adenine (PMEA): III Rat and mouse carcinomas and sarcomas. Folia Biol. 39, 311–314.

Otová, B., Sladká, M., Blazek, K., Schramlová, J., Votruba, I., Holy, A., 1993c. Cytostatic effect of 9-(2-phosphonomethoxyethyl)adenine (PMEA): II. Lymphoblastic leukemia in Sprague–Dawley rats. Folia Biol. 39, 142–149.

Otová, B., Zídek, Z., Holý, A., Votruba, I., Sladká, M., Marinov, I., Leskova, V., 1997. Antitumor activity of novel purine acyclic nucleotide analogs PMEA and PMEDAP. In Vivo 11, 163–167.

Ozinsky, A., Underhill, D.M., Fontenot, J.D., Hajjar, A.M., Smith, K.D., Wilson, C.B., Schroeder, L., Aderem, A., 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. Proc. Natl. Acad. Sci. U.S.A. 97, 13766–13771.

Robbins, B.L., Srinivas, R.V., Kim, C., Bischofberger, N., Fridland, A., 1998. Anti-human immunodeficiency virus activity and cellular metabolism of a potential prodrug of the acyclic nucleoside phosphonate 9-*R*-(2-phosphonomethoxypropyl)adenine (PMPA), bis(isopropyloxymethylcarbonyl)PMPA. Antimicrob. Agents Chemother. 42, 612–617.

Trinchieri, G., 1998. Interleukin-12: a cytokine at the interface of inflammation and immunity. Adv. Immunol. 70, 83–243.

Trinchieri, G., 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat. Rev. Immunol. 3, 133–146.

Van Rompay, K.K.A., Cherrington, J.M., Marthas, M.L., Berardi, C.J., Mulato, A.S., Spinner, A., Tarara, R.P., Canfield, D.R., Telm, S., Bischofberger, N., Pedersen, N.C., 1996. 9-[2-(Phosphonomethoxy)propyl]adenine therapy of established simian immunodeficiency virus infection in infant rhesus macaques. Antimicrob. Agents Chemother. 40, 2586–2591.

Van Rompay, K.K.A., Cherrington, J.M., Marthas, M.L., Lamy, P.D., Dailey, P.J., Canfield, D.R., Tarara, R.P., Bischofberger, N., Pedersen, N.C., 1999. 9-[2-(Phosphonomethoxy)propyl]adenine (PMPA) therapy prolongs survival of infant macaques inoculated with simian immunodeficiency virus with reduced susceptibility to PMPA. Antimicrob. Agents Chemother. 43, 802–812.

Van Rompay, K.K.A., Greenier, J.L., Cole, K.S., Earl, P., Moss, B., Steckbeck, J.D., Pahar, B., Rourke, T., Montelaro, R.C., Canfield, D.R., Tarara, R.P., Miller, C.J., McChesney, M.B., Marthas, M.L., 2003. Immunization of newborn rhesus macaques with simian immunodeficiency virus (SIV) vaccines prolongs survival after oral challenge with virulent SIVmac251. J. Virol. 77, 179–190.

Van Rompay, K.K.A., Singh, R., Brignolo, L., Lawson, J.R., Schmidt, K.A., Pahar, B., Canfield, D.R., Tarara, R.P., Bischofberger, N., Marthas, M., in press. The clinical benefits of tenofovir for simian immunodeficiency virus-infected macaques are larger than predicted by its effects on standard viral and immunological parameters. J. Acquir. Immune Defic. Syndr.

Van Rompay, K.K.A., Brignolo, L.L., Meyer, D.J., Jerome, C., Tarara, R., Spinner, A., Hamilton, M., Hirst, L.L., Bennett, D.R., Canfield, D.R., Dearman, T.G., Von Morgenland, W., Allen, P.C., Valverde, C., Castillo, A.B., Martin, R.B., Valerie, F., Samii, V.F., Bendele, R., Desjardins, J., Marthas, M.L., Pedersen, N.C., Bischofberger, N., 2004a. Biological effects of short-term and prolonged administration of 9-[2-(phosphonomethoxy)propyl]adenine (PMPA; tenofovir) to newborn and infant rhesus macaques. Antimicrob. Agents Chemother 48, 1469–1487.

Van Rompay, K.K.A., Singh, R.P., Pahar, B., Sodora, D.L., Wingfield, C., Lawson, J.R., Marthas, M.L., Bischofberger, N., 2004b. CD8+ cell-mediated suppression of virulent simian immunodeficiency virus during tenofovir treatment. J. Virol. 78, 5324–5337.

Villani, N., Calio, R., Balestra, E., Balzarini, J., De Clercq, E., Fabrizi, E., Perno, C.F., Del Gobbo, V., 1994. 9-(2-Phosphonylmethoxyethyl)adenine increases the survival of influenza virus-infected mice by an enhancement of the immune system. Antiviral Res. 25, 81–89.

Zídek, Z., Holý, A., Frankova, D., Otova, B., 1995. Suppression of rat adjuvant arthritis by some acyclic nucleotide analogs. Eur. J. Pharmacol. 286, 307–310.

Zídek, Z., Holý, A., Franková, D., 1997. Antiretroviral agent (*R*)-9-(2-phosphonomethoxypropyl)adenine stimulates cytokine and nitric oxide production. Eur. J. Pharmacol. 331, 245–252.

Zídek, Z., Frankova, D., Holý, A., 1999. Chemokines, nitric oxide and antiarthritic effects of 9-(2-phosphonomethoxyethyl)adenine (Adefovir). Eur. J. Pharmacol. 376, 91–100.

Zídek, Z., Frankova, D., Holý, A., 2000. Macrophage activation by antiviral acyclic nucleoside phosphonates in dependence on priming immune stimuli. Int. J. Immunopharmacol. 22, 1121–1129.

Zídek, Z., Frankova, D., Holý, A., 2001. Activation by 9-(*R*)-[2-(phosphonomethoxy)propyl]adenine of chemokine (RANTES, macrophage inflammatory protein 1a) and cytokine (tumor necrosis factor alpha, interleukin-10 [IL-10], IL-1b) production. Antimicrob. Agents Chemother. 45, 3381–3386.

Zídek, Z., Potmesil, P., Kmoníèková, E., Holý, A., 2003. Immunobiological activity of *N*-[2-(phosphonomethoxy)alkyl] derivatives of N6-substituted adenines, and 2,6-diaminopurines. Eur. J. Pharmacol. 475, 149–159.