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Red blood cells mediated delivery of 9-(2-phosphonylmethoxyethyl)adenine to primary macrophages: efficiency, metabolism and activity against human immunodeficiency virus or herpes simplex virus

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Abstract

Red blood cells (RBC) may act as selective carriers of drugs to macrophages, an important reservoir of viruses such as human immunodeficiency virus (HIV) and herpes simplex virus type 1 (HSV-1). We therefore assessed the incorporation of 9-(2-phosphonylmethoxyethyl)adenine (PMEA, a potent inhibitor of HIV and HSV-1) into RBC, its delivery to macrophages and its activity against HIV or HSV-1. Loading of PMEA in artificially aged opsonized RBC affords significant levels of intracellular PMEA. RBC metabolize PMEA to its active congener PMEA-diphosphate, although with low efficiency. Exposure of macrophages to RBC-encapsulated PMEA inhibits the replication of both HIV and HSV-1 (about 90% inhibition at the highest RBC:macrophages ratios) even if RBC were removed before virus challenge. By contrast, the antiviral activity of free PMEA removed before virus challenge was irrelevant at concentrations up to 150-fold higher than the 50% effective concentration (EC_{50}). Finally, the antiviral effect of RBC-encapsulated PMEA correlates with PMEA levels in macrophages about 500-fold higher than those achieved by free PMEA (at concentrations 10-fold higher than the EC_{50}). The efficacy of RBC-mediated delivery to macrophages of PMEA (and perhaps of compounds with shorter intracellular half-lives) warrants further studies in infectious diseases involving phagocytizing cells as main targets of the pathogen.

Keywords: Erythrocytes; Human immunodeficiency virus; Herpes simplex virus type 1; Macrophages; PMEA

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1. Introduction

Cells of macrophage lineage (monocyte/macrophages, M/M) play a central role in the pathogenesis of infection by human immunodeficiency virus (HIV) (Gendelman and Meltzer, 1990; Embretson et al., 1993; Lipton and Gendelman, 1995). The very limited lifespan of the majority of CD4 lymphocytes infected by HIV (half-life of less than 2 days) (Ho et al., 1995; Wei et al., 1995) suggests that cells different than CD4 lymphocytes, such as M/M, represent the main reservoir of the virus in the body. In addition, M/M can be the target of other viruses, such as herpes simplex virus type 1 (HSV-1), which are able to enhance the replication of HIV (Golden et al., 1992; Fink et al., 1993). M/M coinfected by HIV and HSV-1 and strongly producing both virus progenies have been found in patients (Heng et al., 1994). Hence, therapeutic strategies must consider the importance of the inhibition of infection and replication of HIV in M/M.

The different metabolism and the peculiar functions of M/M, such as phagocytosis, suggest that antiviral drugs can be somewhat selectively delivered to M/M. This would induce (a) selective targeting of drugs to organs and cells of the reticuloendothelial system and (b) minimization of drug-mediated toxic effects upon non-phagocytic cells.

Several attempts have been made in the past to deliver drugs to M/M. Erythrocytes (red blood cells, RBC) are recognized as useful carriers for encapsulation of drugs, enzymes and other molecules, because of the following properties: firstly, they contain large aqueous volume, are biodegradable and have a long lifespan in the circulation; secondly, drug encapsulation in RBC is relatively easy and achieves a relevant yield; thirdly, RBC contain a number of enzymes (kinases, pyrophosphatases, etc.) potentially able to metabolize the encapsulated drugs (Magnani et al., 1989; De Flora et al., 1993); fourthly, their phagocytosis can be enhanced by promoting the clustering of band 3, that is the predominant RBC transmembrane protein that functions as an anion transport system (Wieth and Brahm, 1985; Jennings, 1984). Band 3 is randomly distributed over the RBC membrane and its clustering can be induced by several agents, such as zinc, melittin, acridine orange, phenylhydrazine, diamine, etc. (Lelkes et al., 1986; Lutz et al., 1987; Clague and Cherry, 1989; Low, 1989; Hui et al., 1990). Once the clusters are formed and stabilized by a cross-linking agent, such as bis(sulfosuccinimidyl)suberate (BS_3), they are viewed by the immune system as non-self and opsonized by autologous antibodies (Turrini et al., 1991). Subsequently, the Fc region of the autoantibody is recognized and bound by M/M, which phagocytize the complex antibody–RBC.

Due to the potential advantages of RBC as drug carriers, we assessed in human primary M/M the delivery, antiviral activity and metabolism of RBC-encapsulated 9-(2-phosphonylmethoxyethyl)adenine (PMEA), a promising prototype compound of the family of acyclic nucleoside phosphonates (De Clercq et al., 1986; Balzarini et al., 1989). PMEA is a potent inhibitor of HIV and HSV-1; in addition, it is characterized by an interesting immunomodulatory effect upon natural immunity (natural killer activity and interferon production) (Del Gobbo et al., 1991; Calio' et al., 1994). PMEA has been chosen for its high hydrosolubility, long intracellular half-life, and clinical relevance, since one of its prodrugs, bispivaloyloxymethyl–PMEA (bis-POM–PMEA), is currently under phase-III evaluation in HIV-infected patients (Barditch-Crovo et al., 1995).

2. Methods

2.1. Cells

Human primary M/M have been obtained from normal seronegative blood donors, as previously described in detail (Perno and Yarchoan, 1993). Briefly, peripheral blood mononuclear cells (PBMC) separated over a Ficoll-Hypaque gradient were seeded in plastic 48-well plates (Costar, Cambridge, MA), at 1.8×10^6 cells/ml in RPMI 1640 (Gibco Labs, Gaithersburg, MD) at which 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 20% heat-inactivated, mycoplasma- and endotoxin-free fetal calf serum (all

from Hyclone, Logan, UT) were added. Unless differently specified, this is the medium used in all experiments described in this paper. PBMC were cultured for 5 days, then non-adherent cells were removed by repeated gentle washings with warm medium. Overall experience and selective control for each experiment show that adherent cells obtained with this technique consist of over 95% mature macrophages.

An African green monkey fibroblastoid kidney cell line, Vero, was used for the comparative experiments of infection. This cell line has no phagocytizing capacity and is highly sensitive to the cytopathic effect of HSV-1.

2.2. Virus

A monocytotropic strain of HIV-1, HTLV-II-$I_{Ba\text{-}L}$ (hereinafter called HIV-BaL), and a laboratory-adapted strain of HSV-1, HSV-EB, were used in experiments of infection with HIV-1 and HSV-1, respectively. Both viruses grow and easily replicate in M/M. HSV-1 induces a cytopathic effect already detectable 48 h after virus challenge and found complete after 96–120 h.

2.3. Chemicals

PMEA, PMEA-monophosphate (PMEAp) and PMEA-diphosphate (PMEApp, the active form of PMEA), kindly provided by Dr N. Bischofberger (Gilead Sciences, Foster City, CA), were more than 99% pure by high-performance liquid chromatography (HPLC) analysis. PMEA was dissolved in phosphate-buffered saline (PBS) at high concentration (1 mg/ml) and stored at 4°C until use. PMEAp and PMEApp, used as standards for the evaluation of PMEA metabolism in RBC, were stored in a powder form and dissolved immediately before use. [2,8-^{3}H]PMEA (17 mCi/mol) was purchased from Moravek (Brea, CA).

Ultrapure standards for HPLC analysis were purchased from Sigma (St. Louis, MO) and Boehringer (Mannheim, Germany). Tetrabutylammonium hydroxide was obtained as a 55% solution from Novachimica (Milan, Italy). HPLC-grade methanol was supplied by JT Baker (Phillipsburg, NJ).

2.4. PMEA encapsulation in RBC

Human RBC were obtained from blood of seronegative healthy donors. To decrease experimental variations, RBC and M/M from the same donor were used in each experiment. RBC were washed twice in 0.9% NaCl and then resuspended at 70% hematocrit in the same solution. Subsequently, RBC were dialyzed for 20 min using a tube with a cut-off of 12 kDa against 10 vol. of lysing buffer (10 mM NaH_2PO_4/20 mM glucose/4 mM $MgCl_2$, at pH 7.4) containing 45 mg/ml PMEA (transient lysis). After incubation at 39°C for 10 min, resealing was performed by adding 0.1 vol. of resealing buffer (5 mM adenine, 100 mM inosine, 100 mM sodium pyruvate, 100 mM glucose, 12% NaCl, at pH 7.4) and further incubation at 37°C for 30 min.

2.5. Artificial aging of RBC

After drug encapsulation, RBC were washed twice in saline with 10 mM Hepes and 140 mM glucose at pH 7.4 (buffer A), incubated for 30 min at room temperature in buffer A containing 1 mM $ZnCl_2$ and 1 mM BS_3, and then washed as follows: first washing in buffer A containing 10 mM ethanolamine, second washing in buffer A containing bovine serum albumin (BSA), and third washing in buffer A without additions. Finally, RBC were resuspended at 45% hematocrit in autologous serum, incubated at 37°C for 1 h, and then washed and resuspended in buffer A. All different washings were stored and analyzed by HPLC to assess drug leakage from RBC: such leakage was undetectable after the third washing (i.e. when resealing is complete).

2.6. Assessment of PMEA loading and stability in RBC

PMEA-loaded packed RBC (1 ml) was washed once with 10 mM glucose-supplemented PBS and centrifuged at $1075 \times g$ for 10 min at 4°C. RBC were then deproteinized by adding 1.2 M ice-cold $HClO_4$. Acid cell extracts were centrifuged at $20\,190 \times g$ for 10 min at 4°C, and subsequently neutralized by the addition of 5 M K_2CO_3. After

an additional centrifugation at $20\,190 \times g$ for 3 min at 4°C, samples were filtered through a 0.45-μm HV-Millipore filter and then loaded (200 μl) onto the ion-pairing C-18 column for HPLC analysis.

Separation of PMEA from AMP was performed according to a modification of an HPLC method for determination of several adenine nucleotide derivatives (Lazzarino et al., 1991). Briefly, an Alltima C-18 5-μm particle-size column (Alltech Associates, Deerfield, IL), provided with its own guard column, was equilibrated with a mobile phase (Buffer B) containing 10 mM tetrabutylammonium hydroxide as the pairing reagent, 10 mM KH_2PO_4, 0.25% methanol, and HCl to adjust pH to 7.00. A step gradient was obtained with buffer C containing 2.8 mM tetrabutylammonium hydroxide, 100 mM KH_2PO_4, and 30% methanol, at pH 5.50. Both buffers were prepared daily. Gradient was formed as follows: with buffer B added for 14 min at 100%, 4 min down to 58%, 12 min down to 55%, 8 min down to 25% and 12 min down to 0%. The flow rate throughout chromatographic runs was 1.5 ml/min and the temperature was constantly kept at 21°C by thermostating the column with water-jacketed glassware.

The presence of PMEAp and PMEApp could be detected only after degradation of ADP and ATP (because they coelute with PMEAp and PMEApp, respectively; see Section 3). For this purpose, the periodate treatment of standard solutions and of RBC extracts was carried out according to Garrett and Santi (1979) before HPLC analysis. Quantitative analysis of both standards and samples was performed at 254 nm wavelength. Assignment of peaks in unknown samples was obtained by comparing both retention times and absorption spectra of runs of ultrapure standards. Each peak was subjected to purity criteria by the computer connected to the diode array detector according to the software program supplied by the HPLC manufacturer (Jasco, Tokyo, Japan). Each concentration was calculated by comparing the corresponding standard areas. The assignment of peak at 43.5 min to PMEApp (see Section 3) was established on the basis of comparison of retention time, absorption spectrum and cochromatogram (with PMEApp standard, run in parallel for each experiment after having completely degraded ribonucleotides by the Garrett and Santi procedure).

2.7. Cell infection and virus detection

Before infection, 5-day adherent M/M were exposed to RBC-encapsulated PMEA (immediately after drug loading and aging) at different RBC:M/M ratios for 90 min or 6 h at 37°C in a humidified atmosphere enriched with 5% CO_2. The number of M/M at the time of treatment with RBC was about 10^5 cells/well (as assessed by scraping and counting cells of representative wells), with under 10% variation from experiment to experiment. To prevent potential leakage of PMEA from RBC impairing the quality of the results, RBC were carefully removed from the wells by repeated (at least six) washings with warm medium before infection, and were not readded. At this point, M/M were challenged for 1 h with HIV-BaL (300 tissue culture infectious doses 50% ($TCID_{50}$)), or with HSV-EB (3 plaque-forming units (PFU)/cell). After incubation with the virus, M/M were carefully washed with warm medium to remove excess virus, and then cultured in the same medium as before. Thus, virus challenge of M/M (as well as their ensuing cultivation) was done in the absence of RBC-encapsulated PMEA. The following controls were performed in each experiment: (1) RBC not loaded with PMEA, but treated exactly as RBC-encapsulated PMEA (including transient lysis, aging and opsonization); (2) free PMEA given to M/M for 90 min or 6 h, and carefully removed before virus challenge; (3) free PMEA given to M/M starting from 90 min or 6 h before virus challenge, and maintained throughout the whole experiment (i.e. also after virus challenge); (4) free PMEA + RBC not loaded with PMEA, given to M/M only before virus challenge (that is for 90 min or 6 h); (5) M/M treated neither with PMEA nor with RBC, infected with HIV-1 or HSV-1; (6) M/M mock-treated and mock-infected.

In the case of infection with HIV-1, M/M were fed every 5–6 days with fresh medium. PMEA was readded only if established by the experimen-

tal protocol (see control no. (3) of the previous paragraph), keeping the same concentrations as before. Virus production was assessed in the supernatants usually 14 days after virus challenge by a commercially available enzyme-linked immunosorbent assay (ELISA) kit able to detect HIV *gag* p24 (Abbott Lab., Pomezia, Italy). In selected experiments (data not shown), the inhibition of HIV production was also assessed by virus titration and found very similar to that achieved by the ELISA assay.

In the case of HSV-1 infection, since M/M are sensitive to the cytopathic effect of HSV within 3–4 days from virus challenge, assessment of virus production was performed in the supernatants of M/M by plaque formation assay and by a commercially available ELISA kit able to recognize HSV antigens (Murex Diagnostics, Dartford, UK), starting from 24 h after virus challenge. Data shown in this paper refer to virus production 48 h after virus challenge. Results at 24, 72 and 96 h after virus challenge were superimposable with those obtained at 48 h.

Each experiment was run with primary M/M from a single blood donor; minor variations have been found among different experiments, yet differences among triplicates (or quadruplicates) of the same experiment were below 5% of the average values.

2.8. Assessment of RBC-mediated delivery of PMEA to M/M

For the assessment of PMEA incorporation in M/M treated with RBC, the following procedure based upon the phagocytizing capacity of M/M, was performed. RBC-encapsulated PMEA were incubated with $Na^{51}Cr$ (^{51}Cr) (New England Nuclear, Boston, MA) for 1 h at 37°C, and then extensively washed to remove unincorporated ^{51}Cr. After assessment of their incorporation of ^{51}Cr (by a γ-counter), RBC were added to 5-day adherent M/M at different RBC:M/M ratios, and cultured for an additional 6 h at 37°C. After careful washings (at least six times) to remove non-phagocytized RBC, M/M were detached, counted (in a hemocytometer chamber) and lysed; specific ^{51}Cr incorporation of M/M (counts per min, cpm) was then assessed in a γ-counter. The concentration of PMEA released by RBC within M/M, related to the average number of RBC phagocytized by M/M, was equal to the cpm of M/M minus background of M/M, divided by the number of M/M. Tests were run in sextuplicate; baseline (background) of M/M not exposed to ^{51}Cr-treated RBC never exceeded 10% of cpm incorporated by M/M.

Intracellular levels of PMEA and its metabolites in M/M exposed to free PMEA were assessed as follows. Five $\times 10^6$ M/M were cultured for 6 h in the presence of 0.1 μg/ml (1 μCi/ml) [2,8-^{3}H]PMEA. Cells were then carefully scraped, counted and centrifuged at least three times in a large volume of cold PBS to remove the excess of radiolabeled PMEA. Cell pellets were then dried, exposed to 400 μl of 60% methanol, centrifuged for 10 min at 13 000 rpm, and the supernatants injected in HPLC. Chromatography was performed on a Partisil-SAX-10-radial compression column. Tests were run in duplicate.

3. Results

We first assessed the efficacy of loading and stability of PMEA within RBC. Incorporation of PMEA within RBC, measured after artificial aging and opsonization, averaged 11.5 μg/10^9 RBC (see Fig. 1A); drug leakage from RBC was undetectable in the first 6 h, and was less than 5% over 24 h. This suggests that the leakage of PMEA from RBC (during the 90-min/6-h period of incubation of RBC-encapsulated PMEA with M/M) is not a major factor affecting the antiviral activity of PMEA-loaded RBC in M/M (see below). Cellular viability after transient lysis and artificial aging was more than 95% and stable over at least 48 h. Thus, autologous RBC can be loaded with marked concentrations of PMEA and maintain their viability over time.

Further analysis was performed on RBC-encapsulated PMEA extracts by the Garrett and Santi periodate procedure to assess whether PMEA metabolites might be present within RBC. For this purpose, a standard solution of ultrapure PMEAp and PMEApp, treated by periodate ex-

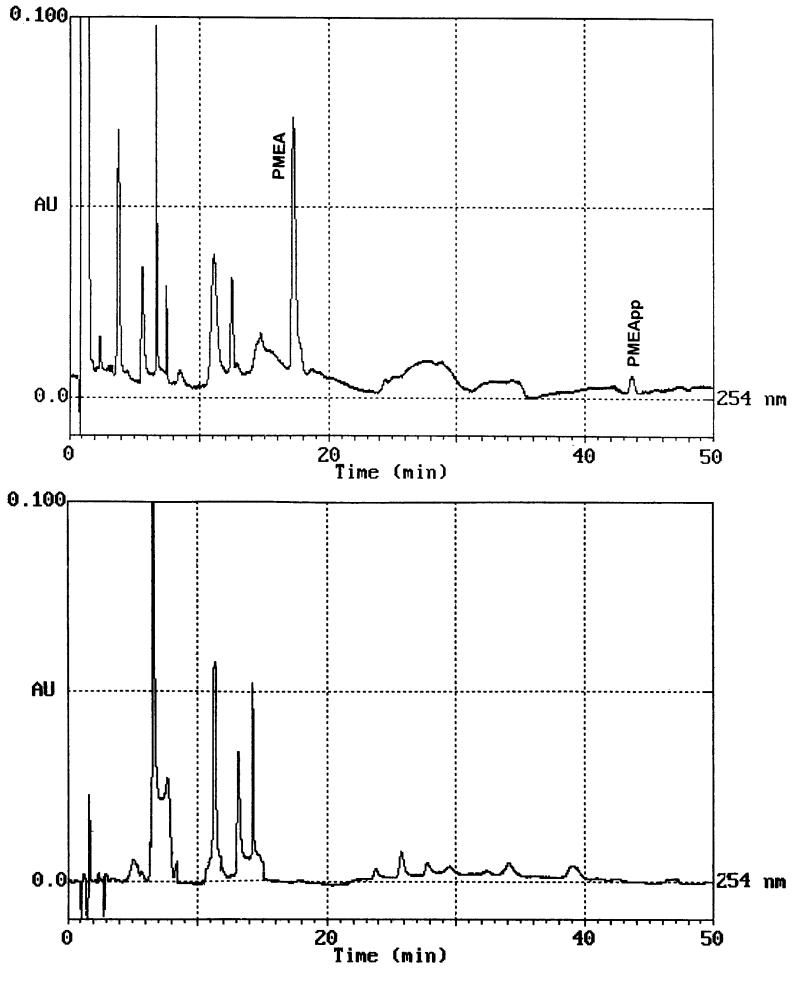

Fig. 1. Incorporation and metabolism of PMEA in erythrocytes.

Panel A. HPLC diagram of erythrocytes loaded with PMEA. Assessment after periodate treatment according to Garrett and Santi (1979). The concentrations of PMEA and of PMEApp within RBC are 11.5 ± 3.4 $\mu g/10^9$ and 0.13 ± 0.04 $\mu g/10^9$ RBC respectively.

Panel B. HPLC diagram of control erythrocytes not loaded with PMEA, but treated, aged, and opsonized exactly as RBC loaded with PMEA, after periodate treatment according to Garrett and Santi (1979).

The figure represents a typical experiment of four. The same peak at 43.5 min was present in all 4 experiments only in cellular extracts obtained from PMEA-loaded RBC, and not in samples from RBC not loaded with PMEA, but treated exactly as the loaded ones. A co-chromatogram was performed with PMEApp standard added to the sample [before Garrett and Santi (1979) procedure] at concentrations capable of doubling the peak area of PMEApp.

actly as RBC-encapsulated PMEA lysates, was run as control: they showed elution times of 32 and 43.5 min, respectively (data not shown). A typical HPLC chromatogram obtained from periodate-treated RBC lysates (to eliminate ADP and ATP that coelute with PMEAp and PMEApp, respectively) is reported in Fig. 1A. A peak eluting at 43.5 min, not degradable by periodate treatment and with the characteristics of PMEApp, was detected in lysates of PMEA-containing RBC (Fig. 1A), but not in RBC not containing PMEA (and treated in the same manner) (Fig. 1B). The amount of PMEApp within RBC averaged 0.13 $\mu g/10^9$ RBC, that is about 90-fold lower than the levels of unmetabolized parent PMEA (see above). Interestingly, no de-

Table 1
Anti-HSV-1 activity of free PMEA in macrophages

[Free PMEA]	% Virus inhibition compared to controls	
	Maintained throughout the experiment[a] (90 min/6 h)	Removed before infection (90 min/6 h)
1.5 μg/ml	>99.9/>99.9	0/0
0.15 μg/ml	71.5/76.7	0/0
0.015 μg/ml	54.5/56.3	0/0

Assessment of virus production was performed 48 h after virus challenge. Typical experiment of four, run in quadruplicate.
[a]Treatment started 90 min/6 h before virus challenge, and continued afterwards.

tectable levels of the intermediate metabolite PMEAp could be found in RBC extracts. Thus, RBC can be considered not only as a carrier of PMEA, but also as cells able to metabolize PMEA toward its active form. This may conceivably affect the antiviral efficacy in M/M of PMEA delivered by RBC against both HSV-1 and HIV.

Table 1 shows the antiviral effect of PMEA (free or RBC-encapsulated) against HSV-1 in M/M. Consistent with previous results (Perno et al., 1996), free PMEA maintained in culture throughout the whole experiment potently inhibits HSV-1, with an EC_{50} of about 0.01 μg/ml; by contrast, no virus inhibition whatsoever could be achieved by free PMEA, even at the highest concentrations tested (that is more than 100-fold higher than the EC_{50}), if the drug was added for 90-min/6-h and then removed before virus challenge (exactly as it was for RBC-encapsulated PMEA). Thus, it is conceivable that the pretreatment of M/M with free compound does not allow concentrations of PMEA to be reached which are sufficiently high and/or maintained long enough to exert an antiviral effect.

It is worth noting that the treatment with control RBC (i.e. treated with transient lysis, aging and opsonization, but not loaded with PMEA) inhibits the replication of HSV-1 in M/M by about 45% at the three highest RBC:M/M ratios tested (i.e. 500:1, 100:1 and 20:1), and only slightly less at the lowest RBC:M/M ratio (4:1) (Table 2). Nevertheless, RBC-encapsulated PMEA at the highest RBC:M/M ratio (500:1) induced a consistent inhibition of virus replication in M/M (average of about 84% compared with control M/M, $P < 0.02$ compared with RBC alone) even upon a short pretreatment of 90 min. Lower PMEA-loaded RBC:M/M ratios induced a less potent inhibitory effect upon virus replication (other than that induced by unloaded RBC per se).

When we increased to 6 h the time of incubation of RBC-encapsulated PMEA with M/M before virus challenge, we found that free PMEA, RBC alone and RBC-encapsulated PMEA, gave results similar to those achieved with a 90-min incubation time (Tables 1 and 2). In particular,

Table 2
Inhibition of HSV-1 production in M/M treated with opsonized RBC before virus challenge

Ratio RBC:M/M	[PMEA] in RBC (μg/ml)	% Virus inhibition compared to controls	
		Unloaded RBC (90 min/6 h)	RBC loaded with PMEA (90 min/6 h)
500:1	0.565	42.7/44.5	83.7/89.5
100:1	0.132	47.2/49.1	60.9/63.8
20:1	0.037	48.2/46.3	53.8/43.6
4:1	0.0049	34.8/44.8	36.4/47.2

RBC have been removed before virus challenge (see Materials and Methods).
Assessment of virus production was performed 48 h after virus challenge. Virus titer in the supernatants of control M/M was 4500 PFU/ml (average of 6 wells).
Typical experiment of two, run in quadruplicate (controls in sextuplicate).

RBC not loaded with PMEA induced less than 50% virus inhibition at all RBC:M/M ratios tested, while RBC loaded with PMEA caused a marked inhibition of HSV-1 replication (about 90%) at the highest ratio (500:1) (Table 2). Free PMEA removed before virus challenge was still completely inactive (Table 1). As additional control, M/M were exposed to unloaded RBC together with free PMEA before virus challenge. Results were identical to those obtained with unloaded RBC (data not shown). Overall results suggest that RBC-mediated delivery of PMEA has a limited but marked effect upon HSV-1 replication in M/M under experimental conditions at which high concentrations of free PMEA are completely inactive; our results also indicate that RBC-mediated drug delivery is not markedly affected by increasing the incubation time (at least within the incubation range used in these experiments).

In parallel experiments, we tested the efficacy of RBC-encapsulated PMEA in Vero cells, a fibroblastoid cell line without phagocytizing capacity. No detectable effect against virus replication could be found either with RBC-encapsulated PMEA or with RBC alone (data not shown).

To validate our results, we assessed the antiviral activity of RBC-mediated drug delivery to M/M challenged with HIV. As shown in Table 3, and consistent with previous results (Balzarini et al., 1991a), free PMEA maintained throughout the culture potently inhibited HIV replication ($EC_{50} < 0.015$ μg/ml), while free PMEA removed before virus challenge induced a very limited inhibition of virus replication only at concentrations at least 100-fold higher than the EC_{50} value. As was seen in the case of HSV-1, RBC alone (i.e. not loaded with PMEA) induced an approximately 40% inhibition of HIV replication at all RBC:M/M ratios tested. Nevertheless, RBC-encapsulated PMEA afforded a marked inhibition of HIV replication not only at the 500:1 RBC:M/M ratio (about 94%), but also at the 100:1 ratio (about 77%) ($P = 0.01$ and 0.03, respectively, against unloaded RBC). Thus, the anti-HIV effect of RBC-encapsulated PMEA was even more sustained than that found for HSV-1, and confirmed our observation that inhibition of virus replication can be achieved by RBC-mediated delivery of PMEA under experimental conditions that afford no substantial activity of free PMEA.

Table 3
Inhibition of replication of HIV in M/M treated with opsonized RBC for 6 h before virus challenge

Ratio RBC:M/M	% Virus inhibition compared to controls	
	Unloaded RBC	RBC loaded with PMEA
500:1	42	94*
100:1	45	77.2**
20:1	43.1	54.7
4:1	38.7	47.6
PMEA without RBC (μg/ml)	Maintained throughout the experiment	Removed after 6 h
1.5	>99.9	12
0.15	86	0
0.015	62.2	0

Assessment of virus production was performed 7 days after challenge with HIV-1.
Typical experiment of two, run in quadruplicate (controls in sextuplicate).
*$P = 0.01$ and **$P = 0.03$ against unloaded RBC.

In a final set of experiments, we assessed whether the antiviral effect of RBC-encapsulated PMEA is related to an increased intracellular concentration of PMEA in M/M. We compared the intracellular concentrations of PMEA ob-

Table 4
Intracellular concentrations of PMEA within M/M

M/M treated with RBC-encapsulated PMEA:	69.4 pmol/10^6 M/M[a]
M/M treated with free PMEA:	0.14 pmol/10^6 M/M[b]

[a]Obtained after incubation of M/M with PMEA-encapsulated RBC at a 1:500 ratio. Transformed in μg/ml, this value roughly corresponds to an intracellular (within M/M) concentration of PMEA of 0.013 μg/ml. The intracellular concentrations of PMEA in M/M were 29.7 and 8.5 pmol/10^6 M/M when these cells were exposed to RBC at ratios of 100:1 and 20:1 respectively.
[b]Obtained after incubation of M/M with 0.1 μg/ml PMEA (that is $\geq$10 fold the EC_{50} of PMEA in M/M infected with HIV or HSV-1).

tained by treating M/M with RBC-encapsulated drug or with free drug. Table 4 shows that the treatment of M/M with 0.1 μg/ml free drug afforded intracellular levels of PMEA of about 0.14 pmol/10^6 M/M. By contrast, treatment of M/M with RBC-encapsulated PMEA at RBC:M/M ratios of 500:1, 100:1 and 20:1 afforded concentrations of PMEA within M/M of 69.4, 29.7 and 8.5 pmol/10^6 M/M, respectively, i.e. concentrations up to 500-fold higher than that achieved by 0.1 μg/ml free PMEA (already $\geq$10-fold higher than the EC_{50} of PMEA against both HIV and HSV-1 in M/M). Under these experimental conditions, no cellular toxicity was detected in M/M treated with either RBC-encapsulated PMEA or with free PMEA. As a control, PMEA levels were undetectable in fibroblastoid Vero cells after exposure to RBC-encapsulated PMEA (data not shown). This latter result is in agreement with the lack of antiviral activity of RBC-encapsulated PMEA in these non-phagocytizing fibroblastoid cells.

4. Discussion

The results reported in this paper show that PMEA removed before virus challenge has no marked residual activity in M/M infected with HIV or HSV-1: this occurs despite the very long intracellular half-life (about 18 h) of the active moiety of PMEA, PMEApp (Balzarini et al., 1991b), one of the longest among nucleoside analogs, the class to which belong the majority of drugs approved for the therapy of HIV infection. By contrast, PMEA encapsulated within RBC inhibits both HIV and HSV-1 in M/M even if removed before virus challenge. This antiviral effect is related to the ability of RBC to selectively deliver PMEA to M/M, to induce high intracellular concentrations of PMEA, and/or to metabolize PMEA toward its active form PMEApp. Such delivery of PMEA is highly efficient, since it occurs at concentrations of PMEA in RBC-containing medium that are similar, or even lower, than those present in medium containing free PMEA. Thus, RBC-mediated drug delivery affords an antiviral activity of PMEA under conditions that do not allow any marked inhibition of virus replication by high concentrations of free drug. This suggests the potential relevance of RBC-mediated delivery not only of PMEA, but also of other antiviral compounds, particularly those characterized by a relatively short intracellular half-life.

In contrast to liposomes, RBC are live carriers, rich in enzymes able to metabolize nucleosides and their analogs, as already shown for ddC (Magnani et al., 1989; De Flora et al., 1993). Thus, our finding that PMEA is metabolized to its diphosphate active form in RBC is not entirely surprising. We do not have any evidence regarding the type of enzyme(s) involved in the activation of PMEA in RBC: one hypothesis is based upon recent results, showing that both mitochondrial AMP kinase and 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase are able to phosphorylate PMEA in various cell types (Balzarini and De Clercq, 1991; Robbins et al., 1995). Their low phosphorylating efficiency suggest that other enzymatic pathways may be more relevant in the phosphorylation of PMEA in cells such as lymphocytes and M/M; however, the limited efficiency of phosphorylation of PMEA in RBC, together with the substantial levels of PRPP synthetase found in these cells (Giacomello and Salerno, 1991; Sakuma et al., 1991), suggest that this enzyme may play a role in the metabolism of PMEA within RBC.

It is interesting to note the limited antiviral effect of RBC-encapsulated PMEA at the lowest RBC:M/M ratios, even if such treatment affords concentrations of PMEA in M/M still greater than those obtained by free PMEA. One possible explanation is that, due to the complexity of the process of phagocytosis (Silverstein et al., 1989), PMEA delivered by RBC may be somewhat compartmentalized in the cytoplasm of M/M, and then slowly released as a free moiety. In this case, low RBC:M/M ratios may not be sufficient to let PMEA reach intracellular concentrations able to inhibit virus replication. Future work will be dedicated to assessing this hypothesis.

Another point worth discussing is the surprising finding of a consistent effect of unloaded RBC upon the replication of both HIV and HSV-1 in M/M. At least in our case, this antiviral effect was not due to a sterical inhibition of HSV-1 (and

HIV) binding upon M/M surface, since RBC were carefully removed before viral challenge (see Section 2). To further confirm this, in other experiments (not reported in this paper) RBC were removed only after the hour of virus adsorption (i.e. after virus challenge), with identical results. The reasons for this antiviral status induced by RBC in M/M are not clear, yet it can be hypothesized that the phagocytosis of RBC induces an activation of M/M functions and/or the production of certain cytokines (such as interleukin-10, a well known inhibitor of HIV replication in M/M) (Borghi et al., 1995) which in turn make M/M less susceptible to the infection by these (and perhaps other) viruses. This would be consistent with the observation of Piedimonte et al. (1993) that, in M/M chronically infected by HIV, the phagocytosis of latex beads or opsonized RBC, as well as the cellular activation mediated by phorbol esters (which mimics the pathway of phagocytosis), markedly increases superoxide anion production, and decreases virus release from infected cells (Piedimonte et al., 1993). Further studies are required to clarify this point.

The potential relevance of RBC-mediated delivery of antiviral drugs to macrophages is still a matter of investigation. Recent studies conducted in murine models have shown that opsonized, artificially aged RBC administered in vivo circulate in the body with a half-life about 3-fold lower than that of normal RBC, and localize in reticuloendothelial organs, with preference for liver, spleen and lymph nodes (Rossi et al., 1993). Thus, such a therapeutic approach affords a selective drug targeting to cells (i.e. M/M) localized in organs whose drug concentration is not necessarily consistent with that achieved in plasma. Indeed, recent evidence shows that the concentrations of anti-HIV drugs in lymph nodes and in the central nervous system are substantially lower than those found in plasma (Yarchoan et al., 1988; Hartman et al., 1990; Dudley, 1995; Manouilov et al., 1995). Under these conditions, the administration of drug doses able to achieve marked (i.e. active) levels in the plasma may not be sufficient to reach similar concentrations in tissues, such as lymph nodes, recognized as the most relevant milieu where HIV both replicates and infects new target cells (Embretson et al., 1993; Pantaleo et al., 1993). Recent experimental evidence suggests that RBC-mediated delivery might overcome this problem. Indeed, Magnani et al. (1992) have shown that RBC-mediated delivery of 2′,3′-dideoxycytidine-triphosphate (ddCTP) induces a dramatic decrease of the weight of lymph nodes of mice experimentally infected with LP-BM5 (a retrovirus that causes an immunodeficiency similar to human AIDS), and a marked delay of the development of the disease; this clinical effect came together with a decreased expression of virus antigen in M/M far more pronounced than in lymphocytes (Magnani et al., 1992). Similar results have been obtained in cats infected with feline immunodeficiency virus (Magnani et al., 1994). Thus, at least in these experimental models, RBC-loaded drugs reach lymph nodes, selectively target M/M, and exert a marked antiviral effect. This might suggest the potential advantage of a selective drug delivery to M/M via RBC (with the purpose of inhibiting virus replication, and/or of killing such chronically infected cells), combined with treatment with other antivirals administered systemically as free compounds, and thus able to reach other (non-phagocytizing) cells targeted by the pathogen. In the case of HIV, this combined therapy may afford a better drug targeting towards the two cell types recognized as major targets of HIV in the body.

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