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PHOSPHONOACETATE INHIBITION OF DEOXYRIBONUCLEIC ACID SYNTHESIS IN INTACT AND PERMEABLE EUKARYOTIC CELLS

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Abstract—Phosphonoacetate was evaluated as an inhibitor of DNA synthesis in L cells, L1210 cells and phytohemagglutinin-stimulated human lymphocytes. L cells were most sensitive and lymphocytes least sensitive to inhibition by phosphonacetate. L cells showed rapid uptake of [^{14}C]phosphonoacetate compared to much slower uptake by L1210 cells and human lymphocytes. The rate of uptake in the three cell types correlated with the degree of inhibition of DNA synthesis. When the drug was studied in permeabilized cells, the DNA synthetic enzymes in all three cell types showed the same susceptibility to inhibition by phosphonoacetate. Thus, the differences in phosphonoacetate inhibition of DNA synthesis in intact L cells, L1210 cells and human lymphocytes appear to be due to differences in drug uptake rather than to any differences in sensitivity to its inhibitory action.

Phosphonoacetate inhibits the DNA polymerases of certain DNA viruses, and this inhibition appears to account for its ability to prevent replication of these viruses [1–6]. While phosphonoacetate was at first thought to be a specific inhibitor of the viral polymerases, subsequent studies showed that at higher concentrations it was an effective inhibitor of some DNA polymerases from eukaryotic sources [1–8]. Despite its ability to inhibit eukaryotic DNA polymerases, phosphonoacetate has shown a variable ability to inhibit DNA synthesis in intact cells. In our studies with L cells, L1210 cells and phytohemagglutinin (PHA)-stimulated human lymphocytes, treatment with equimolar concentrations of phosphonoacetate resulted in different degrees of inhibition of DNA synthesis in the different cell types. The present study was conducted to determine whether the different degrees of inhibition were due to differences in uptake of the agent or to intrinsic differences in the susceptibilities of the DNA replication systems. To evaluate the latter possibility, we used a technique by which replicative DNA synthesis can be studied in cells rendered permeable to exogenously supplied nucleotides and other small molecular weight compounds [9, 10]. This allowed us to test directly the effects of similar concentrations of phosphonoacetate on the DNA synthesis complexes functioning on their intrinsic DNA templates in each of the different cell types.

METHODS

Nucleotides were purchased from P-L Biochemicals, Milwaukee, WI; α-modified Eagle's medium and fetal calf serum were purchased from Flow Laboratories, Rockville, MD. Serum was heated at 56° for 30 min before use in media. [Me-^{3}H]Thymidine (sp.act. 6.7 Ci/m-mole), [Me-^{3}H]thymidine 5′-triphosphate, tetrasodium salt (sp. act. 40–60 Ci/m-mole), and protosol were purchased from the New England Nuclear Corp., Boston, MA. PHA-P was purchased from Difco Laboratories, Detroit, MI, and was used to prepare leukoagglutinating PHA (L-PHA) as described previously [11]. Dextran, Ficoll, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from the Sigma Chemical Co., St. Louis, MO. GF/C filter discs were purchased from the Reeve Angel Co., Clifton, NJ. Swinnex 13 filters and EGWPO2500 filter discs were from the Millipore Corp., Bedford, MA. Sodium phosphonoacetate and [^{14}C]phosphonoacetate (sp. act. 15.73 μCi/mg) were gifts from Abbott Laboratories, Chicago, Il. Solutions of phosphonoacetate to be tested in cultures of intact cells were prepared in 0.9% NaCl; and sterilized through Swinnex 13 filters. Solutions of phosphonoacetate for studies in permeable cells were prepared in 0.01 M Tris/HCl and adjusted to a final pH of 7.8.

L cells, a continuous line of mouse fibroblasts, and L1210 cells, a line of mouse lymphocytic leukemia cells, were maintained in suspension culture in α-modified Eagle's medium buffered at pH 7.2 with 25 mM HEPES and supplemented with 10% fetal calf serum (α-MEM–HEPES–10% FCS). Normal human peripheral blood lymphocytes were isolated from difibrinated, dextran sedimented blood by isopycnic centrifugation on Ficoll–Hypaque gradients as described previously [12, 13]. The lymphocytes were cultured in α-MEM–HEPES–10% FCS at 5×10^5 cells/ml in 20 ml volumes in 150 cm T flasks. Purified L-PHA was added at the beginning of lymphocyte culture at a concentration of 1.7 μg/ml. This concentration has been determined previously to produce optimal stimulation of DNA synthesis. Lymphocytes were used for studies of the phosphonoacetate effect on the third day after PHA stimulation, which was the day of peak DNA synthesis.

DNA synthesis in intact cells was measured by pulsing 2-ml aliquots of cell suspension containing 3–5×10^5 cells/ml with 1 μCi [^{3}H]thymidine for 30 min at 37°. The reactions were terminated by adding 7 ml of

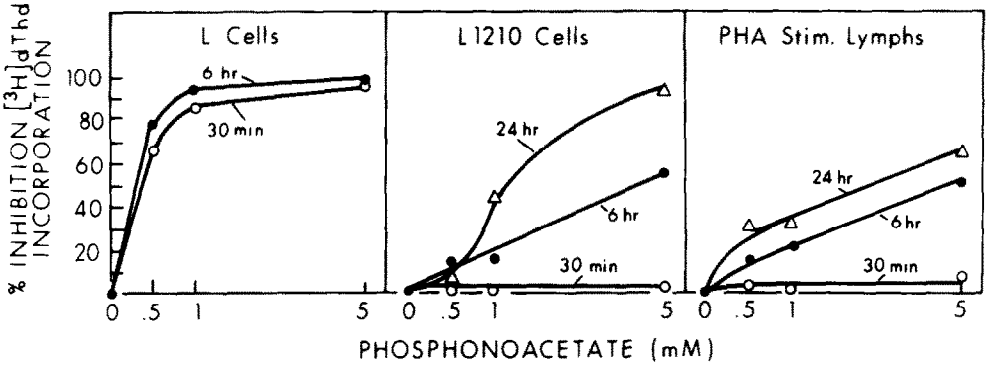

Fig. 1. Effects of phosphonoacetate on DNA synthesis in intact cells. L cells (left panel), L1210 cells (middle panel), or PHA-stimulated human lymphocytes (right panel) were incubated with phosphonoacetate at the indicated concentrations for 30 min (○), 6 hr (●), or 24 hr (△). At the end of each incubation period, 2-ml aliquots of treated and untreated cell suspensions were pulsed with 1 μCi [^{3}H]thymidine for 30 min at 37°. Cell pellets were prepared, as described in methods, to determine incorporation of [^{3}H]thymidine into acid precipitable counts. Per cent inhibition is calculated relative to control, untreated cultures performed at each time point. The amount of [^{3}H]thymidine incorporated during the first 30-min pulse by the control cells was 129×10^3 dis./min/10^5 L cells, 115×10^3 dis./min/10^5 L1210 cells, and 23×10^3 dis./min/10^5 lymphocytes.

cold 0.9% NaCl to each tube and collecting the cell pellets by centrifugation at 2200 *g* for 7 min at 4°. Cell pellets were treated with cold 5% trichloroacetic acid, sonicated, collected on GF/C discs, washed four more times with trichloroacetic acid, then ethanol, and dried. The discs were treated with 0.5 ml protosol overnight; then radioactivity was counted after the addition of 10 ml of Toluene scintillation fluid (containing 6 g PPO and 50 mg POPOP per liter).*

Cell permeabilization was performed as described previously [8–10]. Cells were collected from growth media by centrifugation for 10 min at 4°, 1000 *g*. The L cells were suspended at 2×10^6 cells/ml in buffer composed of 0.01 M Tris/HCl (pH 7.8), 0.25 M sucrose, 1 mM EDTA, 30 mM 2-mercaptoethanol and 4 mM $MgCl_2$, and incubated for 15 min at 4°. The cells were centrifuged again and resuspended at 2.5×10^7 cells/ml in the same buffer. Sucrose was omitted from the buffers used to permeabilize the L1210 cells and human lymphocytes. Following this treatment, the cells were all permeable, as shown by uptake of Trypan Blue [14].

The reaction mixture for DNA synthesis contained 0.1 M HEPES (pH 7.8), 0.02 M $MgCl_2$, 0.21 M NaCl, 15 mM ATP, 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, and 2.3 μM [Me-^{3}H]dTTP (sp. act. 43.7×10^6 cpm/nmole) [8, 10].

Fifty-μl aliquots containing 1.25×10^6 permeable cells were combined with 25 μl of the DNA synthesis mixture in an ice–water bath. The reactions were started by transferring reaction tubes to a 37° water bath. The reactions were terminated by addition of an excess of 10% trichloroacetic acid–2% sodium pyrophosphate. The precipitates were sonicated, collected and prepared for scintillation counting on filter discs as described above. Results are expressed as the means of triplicate assays which showed less than 10 percent variation.

* PPO = 2,5-diphenyloxazole; and POPOP = 1,4-bis-[2-(5-phenyloxazolyl)]-benzene.

For studies of [^{14}C]phosphonoacetate uptake, the L and L1210 cells were in mid log phase growth and the lymphocytes were on the third day of PHA stimulation. The cells were collected by centrifugation at 1000 *g* for 5 min at 25°, and then resuspended at 2-4 $\times 10^5$ cells/ml in fresh α-MEM–HEPES–10% FCS. The suspensions were incubated in spinner flasks at 37°. [^{14}C]phosphonoacetate was added to each flask at a final concentration of 0.5 mM. Duplicate 1-ml aliquots were removed from each flask before addition of [^{14}C]phosphonoacetate and at sequential time intervals thereafter. The cells were collected immediately by filtration with suction onto EGWPO2500 Millipore filters. They were washed five times with 5-ml portions of cold α-MEM–HEPES–10% FCS containing 0.5 mM carrier phosphonoacetate and then washed twice with 10 ml of cold saline. This washing procedure took about 1 min/sample; preliminary experiments demonstrated that no exchange of intracellular [^{14}C]phosphonoacetate occurred during these washings. The discs were dried, solubilized with 0.5 ml protosol and counted in 10 ml of Brays scintillation fluid [15]. Results are expressed as the mean of duplicate assays which showed less than 10 per cent variation.

RESULTS

The effects of phosphonoacetate on DNA synthesis in L cells, L1210 cells and PHA-stimulated human lymphocytes are shown in Fig. 1. Within 30 min of the addition of 0.5 mM phosphonoacetate to cultures of L cells, DNA synthesis was inhibited by 67 per cent. Five millimolar phosphonoacetate inhibited DNA synthesis by 96 per cent within the same time period. Only a slight increase in the inhibition of DNA synthesis occurred when the L cells were incubated in these concentrations of phosphonoacetate for 6 hr or more. Phosphonoacetate had essentially no effect on L1210 cells or PHA-stimulated human lymphocytes during a 30-min incubation period. After a 6-hr incubation

period, 0.5 mM phosphonoacetate caused only 15 per cent inhibition of DNA synthesis in the L1210 cells and PHA-stimulated lymphocytes. After 6 hr of incubation in 5 mM phosphonoacetate, DNA synthesis was inhibited by only 57 per cent in the L1210 cells and by 53 per cent in the PHA-stimulated human lymphocytes. When the L1210 cells were incubated with 5 mM phosphonoacetate for 24 hr, DNA synthesis was inhibited by 93 per cent. When the PHA-stimulated human lymphocytes were incubated with 5 mM phosphonoacetate for 24 hr, DNA synthesis was inhibited by 63 per cent. Thus, phosphonoacetate differed markedly in its ability to inhibit DNA synthesis in the different cell types, except when the cells were incubated in phosphonoacetate for prolonged periods.

To determine whether these differences could be accounted for by differences in uptake of phosphonoacetate, the different cell types were incubated in [^{14}C]phosphonoacetate and the accumulation of intracellular radioactive material was assayed over the next 24 hr. The L cells showed the fastest uptake of [^{14}C]phosphonoacetate, reaching a maximum concentration of intracellular radioactive material in the first 3 hr of the incubation (Fig. 2). In contrast, the PHA-stimulated lymphocytes and the L1210 cells showed a much slower uptake. After 6 hr of incubation, the amount of radioactive material in the L1210 cells and in the PHA-stimulated lymphocytes was less than 25 percent of that taken up by the L cells. These different uptakes correlate well with the relative degrees of inhibition—that is, the L1210 cells and PHA-stimulated lymphocytes showed slight inhibition of DNA synthesis and slight uptake of [^{14}C]phosphonoacetate. L cells showed considerably more inhibition of DNA synthesis and greater uptake of radioactive phosphonoacetate. The intracellular concentration of phosphonoacetate, calculated from measurements of the intracellular radioactivity and the packed cell volume was approximately 150 μM in the L cells at the 3-hr incubation point. This is well within the concentration range of the apparent inhibitory constants, 28–200 μM, determined

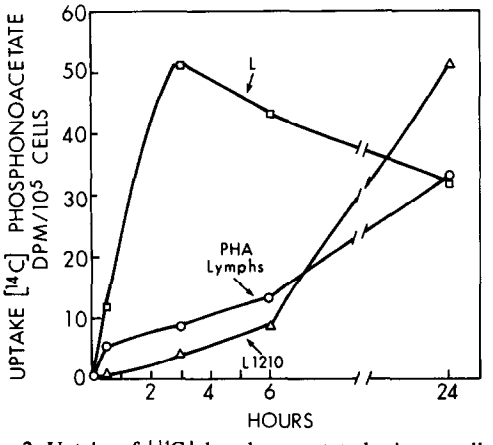

Fig. 2. Uptake of [^{14}C]phosphonoacetate by intact cells. L cells ( ), L1210 cells (△) in mid log phase growth, or PHA-stimulated human lymphocytes (○) on day 3 of culture were suspended in fresh media containing 0.5 mM [^{14}C]phosphonoacetate. Duplicate aliquots were removed from each suspension at the indicated times and assayed for intracellular [^{14}C]phosphonoacetate, as described in Methods.

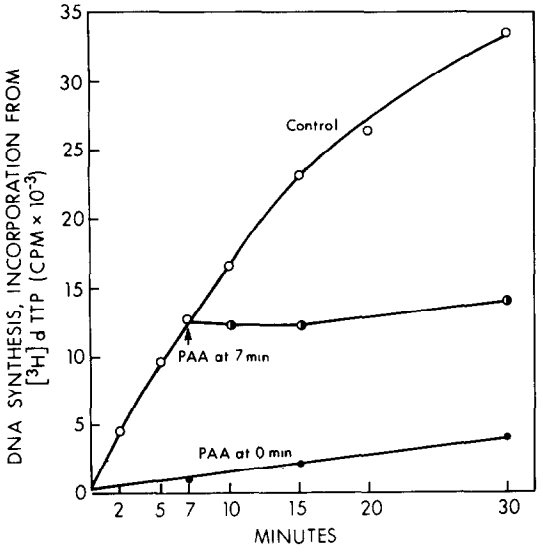

Fig. 3. Effects of phosphonoacetate on DNA synthesis in permeabilized PHA-stimulated human lymphocytes. Reaction mixtures were composed of 50 μl containing 1.25×10^6 permeabilized cells plus 25 μl of DNA synthesis mix containing 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP and 2.3 μM [Me-^{3}H]dTTP (sp. act. 43.7×10^6 cpm/nmole), as detailed in methods. Incorporation from [^{3}H]dTTP into acid precipitable material was measured at 37°. Permeable cells with no phosphonoacetate (○——○); 10 mM phosphonoacetate added just before reactions were started (●——●) and 10 mM phosphonoacetate added to permeable cells after a 7-min incubation at 37° (◑——◑).

for phosphonoacetate with several eukaryotic DNA polymerases [7, 8]. This correlation strongly suggests that the extent of phosphonoacetate uptake accounts for the degree of inhibition of DNA synthesis demonstrated in the three cell types.

Studies were then conducted with cells made permeable to exogenous nucleotides to determine whether the replicative DNA synthesis complexes in these three cell types showed differences in their susceptibilities to inhibition by phosphonoacetate. We have demonstrated that the DNA synthesized in permeable cells is the product of semiconservative replicative synthesis and that it occurs as extensions of replication sites that were active *in vivo* just before the cellls were permeabilized [9, 10]. The activity measured in the permeable cells fluctuates with the replicative activity of the intact cells [9, 16]. Thus, the permeable cell system measures the activity of the replicative DNA synthesis complex functioning on its intrinsic DNA template. Since in the permeable cells agents can be supplied to the replication complex without problems of transport or pool size, this technique provides a rapid assay system to determine the direct effect of phosphonoacetate on the DNA replication complexes of the three different cell types.

The kinetics of DNA synthesis in the permeabilized PHA-stimulated human lymphocytes are shown in Fig. 3. When phosphonoacetate was added to the permeable cells at the beginning of the reaction, there was a marked inhibition of DNA synthesis. When it was added to a series of permeable cell reactions after a 7-min incubation, there was a prompt arrest of DNA synthesis. Thus, in the permeable cells, where exoge-

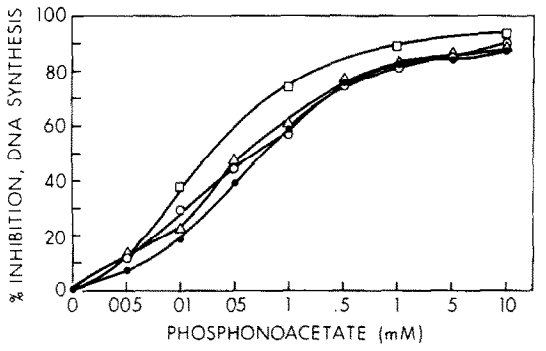

Fig. 4. Concentration dependence of phosphonoacetate inhibition of DNA synthesis in permeabilized cells. L cells (□), L1210 cells (△), and PHA-stimulated human lymphocytes from two different donors (○, ●) were permeabilized and incubated in complete DNA synthesis mix including [^{3}H]dTTP and varying concentrations of phosphonoacetate. Reactions were incubated for 30 min at 37° and incorporation of radioactivity into acid precipitable material was determined, as outlined in Methods. Per cent inhibition was calculated relative to the DNA synthesized by untreated cells in the same permeabilization and incubation.

nously added compounds had immediate access to the replication complex, phosphonoacetate produced drastic inhibition of DNA synthesis. The kinetics of phosphonoacetate inhibition were identical with L cells and L1210 cells.

We then used the permeable cell technique to compare the concentration dependences of phosphonoacetate inhibition of DNA synthesis in the different cell types. Figure 4 shows that phosphonoacetate inhibited DNA synthesis to the same extent in permeable L cells, L1210 cells and in the PHA-stimulated human lymphocytes from two different donors when the permeable cells were exposed to the same concentration of phosphonoacetate.

DISCUSSION

Phosphonoacetate was identified as a potentially useful antiviral agent when it was found to inhibit herpes virus production at a drug concentration that did not interfere with the growth of WI-38 human fibroblasts [1]. It was also found to inhibit production of the Epstein Barr virus at concentrations of drug that did not interfere with the growth of human or marmoset lymphoblastoid cell lines [2]. The antiviral activity of phosphonoacetate appears to be due to its ability to inhibit DNA polymerase and thus prevent virus replication [3]. Using isolated herpes simplex virus DNA polymerase, Mao *et al.* [3, 4] showed that phosphonoacetate inhibited the elongation step of DNA synthesis. At higher concentrations than those used to inhibit virus DNA synthesis, phosphonoacetate inhibits DNA synthesis and replication in many different cell types. Leinbach *et al.* [5] indicated that the inhibition was due to the interaction of the phosphonoacetate with the pyrophosphate binding site of DNA polymerase. Phosphonoacetate has been shown to inhibit the DNA polymerases of vaccinia virus [6], Marek's Disease and herpes virus of turkeys [5], as well as DNA polymerase α from HeLa cells [6], duck embryo fibroblasts [5], CHO cells, calf thymus and PHA-stimulated human lymphocytes [8]. Phosphonoacetate has also been shown to inhibit the DNA polymerases α, β and γ from L1210 cells [7]. However, at the concentrations examined, phosphonoacetate did not affect the DNA polymerases from WI-38 cells [3, 4], duck polymerase β, *Escherichia coli* DNA polymerase I or avian myeloblastosis virus reverse transcriptase [5].

Since phosphonoacetate inhibited the DNA polymerases from HeLa cells but had no apparent effect on the polymerase from WI-38 cells, the suggestion was made that this compound might be a specific inhibitor of DNA polymerases from selected viruses and transformed cells but might not have an effect on the DNA polymerases from normal human cells [17]. In the present study, phosphonoacetate caused less inhibition of DNA synthesis in intact human lymphocytes than it did in intact L cells. However, when the susceptibilities of the DNA replication complexes were examined in permeable cells, the normal human lymphocytes showed the same susceptibility to inhibition from phosphonoacetate as did transformed cell lines. Thus, the varying rates of DNA synthesis inhibition in the three types of intact cells may be due to differences in the rates of drug uptake, as demonstrated in Fig. 2. It is also possible that some differences in drug effects may be due to the concentrations of intracellular metabolites with which phosphonoacetate competes to inhibit DNA synthesis or to some intracellular metabolism of phosphonoacetate itself. In any event, it is apparent that phosphonoacetate can inhibit the DNA replication systems from a variety of cells including normal human lymphocytes and that under appropriate conditions phosphonoacetate can be used to produce rapid inhibition of DNA synthesis in several different eukaryotic systems.

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REFERENCES

1. L. R. Overby, E. E. Robishaw, J. B. Schleicher, A. Rueter, N. L. Shipkowitz and J. C.-H. Mao, *Antimicrob. Agents Chemother.* **6**, 360 (1974).
2. O. Nyormoi, D. A. Thorley-Lawson, J. Elkington and J. L. Strominger, *Proc. natn. Acad. Sci. U.S.A.* **5**, 1745 (1976).
3. J. C-H. Mao, E. E. Robishaw and L. R. Overby, *J. Virol.* **15**, 1281 (1975).
4. J. C-H. Mao and E. E. Robishaw, *Biochemistry* **14**, 5475 (1975).
5. S. S. Leinbach, J. M. Reno, L. F. Lee, A. F. Isbell and J. A. Boezi, *Biochemistry* **15**, 426 (1976).
6. A. Bolden, J. Aucker and A. Weissbach, *J. Virol.* **16**, 1584 (1975).
7. H. S. Allaudeen and J. R. Bertino, *Biochim. biophys. Acta* **520**, 490 (1978).
8. C. L. K. Sabourin, J. M. Reno and J. A. Boezi, *Archs Biochem. Biophys.* **187**, 96 (1978).
9. N. A. Berger and E. S. Johnson, *Biochim. biophys. Acta* **425**, 1 (1976).
10. N. A. Berger, S. J. Petzold and E. S. Johnson, *Biochim. biophys. Acta* **478**, 44 (1977).

11. R. Kornfeld, W. T. Gregory and S. A. Kornfeld, *Meth. Enzym.* **28**, 344 (1972).
12. J. Mendelsohn, S. A. Skinner and S. Kornfeld, *J. clin. invest.* **50**, 818 (1971).
13. N. A. Berger and A. M. Skinner, *J. Cell Biol.* **61**, 45 (1974).
14. E. A. Boyse, L. J. Old and I. Chouroulinkov, in *Methods in Medical Research* (Ed. H. N. Eisen), Vol. 10, p. 39. Yearbook Medical Publishers, Chicago (1964).
15. G. A. Bray, *Analyt. Biochem.* **1**, 279 (1960).
16. N. A. Berger, J. W. Adams, G. W. Sikorski, S. J. Petzold and W. T. Shearer, *J. clin. Invest.* **62**, 111 (1978).
17. L. R. Overby, R. G. Duff and J. C-H. Mao, *Ann. N.Y. Acad. Sci.* **284**, 310 (1977).